PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/16 — A1

(11) International Publication Number: WO 98/37878

(43) International Publication Date: 3 September 1998 (03.09.98)

(21) International Application Number: PCT/US98/03672

(22) International Filing Date: 26 February 1998 (26.02.98)

(30) Priority Data:

08/807,398	27 February 1997 (27.02.97)	US
08/956,320	23 October 1997 (23.10.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/807,398 (CIP)
Filed on	27 February 1997 (27.02.97)
US	08/956,320 (CIP)
Filed on	23 October 1997 (23.10.97)

(71) Applicant *(for all designated States except US)*: GENZYME CORPORATION [US/US]; One Mountain Road, P.O. Box 9322, Framingham, MA 01701-9322 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CHENG, Seng, H. [MY/US]; 50 Wall Street, Wellesley, MA 02181 (US). JIANG, Canwen [CN/US]; 7 Royal Crest Drive #4, Marlboro, MA 01752 (US).

(74) Agent: DUGAN, Deborah, A.; Genzyme Corporation, One Mountain Road, P.O. Box 9322, Framingham, MA 01701-9322 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS AND COMPOSITIONS FOR TREATING CYSTIC FIBROSIS

(57) Abstract

Methods and compositions for treating CF by mobilizing mutant forms of CFTR, which retain at least some functional activity, to the plasma membrane where they can mediate chloride ion transport are disclosed. The preferred agent to be used is deoxyspergualin (DS6).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS AND COMPOSITIONS FOR TREATING CYSTIC FIBROSIS

Background of the Invention

Cystic Fibrosis (CF) is the most common fatal genetic disease in humans (Boat *et al.* (1989), Cystic Fibrosis, In: *THE METABOLIC BASIS OF INHERITED DISEASE*, Scriver, Beaudet, Sly and Valle, eds., McGraw Hill, New York, pp.2649-2860). Based on both genetic and molecular analysis, a gene associated with CF was isolated as part of 21 individual cDNA clones and its protein product predicted (Kerem *et al.* (1989), *Science* 245:1073-1080; Riordan *et al.*(1989), *Science* 245:1066-1073; Rommens *et al.* (1989), *Science* 245:1059-1065).

The product of the CF-associated gene, the cystic fibrosis transmembrane conductance regulator (CFTR), is a protein of approximately 1480 amino acids made up of two repeated elements, each having six transmembrane segments and a nucleotide binding domain. The two repeats are separated by a large, polar, so-called R-domain containing multiple potential phosphorylation sites. Based on its predicted domain structure, CFTR is a member or a class of related proteins which includes the multi-drug resistance (MDR) or P-glycoprotein, bovine adenyl cyclase, the yeast STE6 protein as well as several bacterial amino acid transport proteins (Riordan *et al.*, *supra*; Hyde *et al.* (1990), *Nature* 346:362-365). Proteins in this group, characteristically, are involved in transporting molecules into or out of cells.

CFTR has been postulated to regulate the outward flow of anions from epithelial cells in response to phosphorylation by cyclic AMP-dependent protein kinase or protein kinase C (Riordan *et al.*, *supra*; Frizzell *et al.*, *supra.*; Welsh and Liedtke (1986), *Nature* 322:467; Li *et al.*(1988), *Nature* 331:358-360; Hwang *et al.* (1989), *Science* 244:1351-1353).

Sequence analysis of the CF associated gene has revealed a variety of mutations (Cutting *et al.* (1990a), *Nature* 346:366-369; Cutting *et al.* (1990b), *Am. J. Hum. Genet.* 47:213; Dean *et al.* (1990), *Cell* 61:863-870; Kerem *et al.* (1989), *Science* 245:1073-1080; and Kerem *et al.* (1990), *Proc. Natl. Acad. Sci., USA* 87:8447-8451). Mutations in the gene encoding CFTR result in the synthesis of aberrant variants that are either unstable, mislocalized, or whose Cl^- channel activity is dysfunctional as a consequence of defective regulation or conduction (Welsh and Smith (1993), *Cell* 73:1251-1254). Over 200 different mutations have been described to date, but by far the most prevalent is a deletion of the three nucleotides that encode phenylalanine at position 508 (Phe^{508}) located within the first nucleotide binding domain of CFTR (Tsui, L.C. (1992), *Hum. Mutat.* 1:197-203). The Phe^{508} deletion ($\Delta F508$) is associated with approximately 70% of the cases of cystic fibrosis.

Studies on the biosynthesis (Cheng *et al.* (1990), *Cell* 63:827-834; Gregory *et al.* (1990), *Nature* 347:382-386) and localization (Denning *et al.* (1992), *J. Cell Biol.* 118:551-559) of $\Delta F508$, as well as other CFTR mutants, indicate that many CFTR mutant proteins are not

processed correctly and, as a result, are not delivered to the plasma membrane (Gregory *et al.*, *supra*). These conclusions are consistent with earlier functional studies which failed to detect cAMP stimulated Cl^- channels in cells expressing CFTR ΔF508 (Rich *et al.*, *supra*; Anderson *et al.* (1991), *Science* 251:679-682).

It is believed that the deletion of residue 508 in ΔF508-CFTR prevents the nascent protein from folding correctly, and consequently the variant is recognized by the quality control mechanism present within the endoplasmic reticulum (ER) to select out against misfolded or mutant proteins (Cheng *et al.* (1990), *supra.*; Gregory *et al. supra.*). The mutant ΔF508-CFTR bears carbohydrate structures characteristic of glycosylation at the ER and is eventually degraded. The inability of this mutant protein to exit the ER, to pass through the Golgi where it normally would be fully glycosylated, and traffic to the plasma membrane most likely accounts for the defective Cl^- transport found in CF epithelia harboring this mutation (Quinton, P.M. (1990), *FASEB J.* 4:2709-2727). Studies have shown, however, that ΔF508-CFTR, when presented at the plasma membrane is functional as a cAMP-responsive Cl^- channel (Dalemans *et al.* (1991), *Nature Lond.* 354:526-528; Denning *et al.*, *supra.*; Pasyk and Foskett (1995), *J. Cell. Biochem.* 270:12347-50).

Thus, there is a need in the art for methods and compositions which enable relocation of mislocalized CFTR mutants which retain at least some functional activity (i.e., ΔF508) to the plasma membrane of epithelial cells

where they can effectively mediate chloride ion transport and restore sufficient membrane conductance. The present invention satisfies this need and provides related advantages as well.

Summary of the Invention

The present invention pertains to methods for treating a subject having cystic fibrosis (CF). The methods involve the administration of an effective amount of an agent that facilitates the delivery of the mutant CFTR to the plasma membrane to the subject having CF. The agent interferes with and/or modulates the functioning of molecular chaperone proteins thereby allowing the mutant CFTR protein to escape from the ER, proceed to the plasma membrane and provide functional cAMP-responsive Cl^- channels.

The present invention further pertains to a method for treating a subject's lung epithelia containing a mutant CFTR protein. The method involves contacting a subject's lung epithelia with an agent which interferes with and/or modulates the functioning of molecular chaperone proteins thereby allowing the mutant CFTR protein to escape from the ER, proceed to the plasma membrane and provide functional cAMP-responsive Cl^- channels.

The present invention even further pertains to a method for treating a subject having CF by administering an effective amount of an agent that interferes with and/or modulates the functioning of molecular chaperone

proteins thereby allowing the mutant CFTR protein to escape from the ER, proceed to the plasma membrane and provide functional cAMP-responsive Cl^- channels.

Other aspects of the present invention include therapeutic compositions and packaged drugs for treating subjects having CF. The therapeutic compositions include a therapeutically effective amount of the aforementioned agents, and a pharmaceutically acceptable carrier. The packaged drug includes the aforementioned agents and instructions for administrating the agent for treating subjects having CF.

The present invention further provides methods and compositions for treating CF by mobilizing mutant forms of CFTR, which retain at least some functional activity, to the plasma membrane where they can mediate chloride ion transport are disclosed.

Accordingly, the invention described herein relates to methods and compositions useful for delivering mutant cystic fibrosis transmembrane regulator (CFTR) proteins, which retain at least some functional activity, to the plasma membrane of epithelial cells, where they can mediate chloride ion transport.

The above discussed and many other features and advantages of the present invention will become better understood by reference to the following detailed description when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figures 1A-1E show the effects of DSG on recombinant C127-ΔF508-CFTR cells. Figure 1A shows immunoprecipitation analysis of C127-ΔF508-low cells. Figure 1B shows SPQ analysis using DSG concentrations 10-100μg/ml. Figure 1C shows SPQ analysis following treatment with 10μg/ml DSG, sodium butyrate and growth at lowered temperature. Figure 1D shows SPQ analysis following treatment with 10μg/ml, 20μg/ml and 50μg/ml DSG. Figure 1E summarizes the overall effects concentration of DSG on recombinant C127-ΔF508-CFTR cells at normal and reduced temperature.

Figures 2A and 2B show the results of SPQ analysis of human CF airway epithelial cells following treatment with DSG. Figure 2A shows the shift in fluorescence in cells treated with DSG, growth at lowered temperature and control. Figure 2B shows the percentage of responsive cells following treatment with different concentrations of DSG or growth at lowered temperature.

Figure 3 shows the results of SPQ analysis of DSG treated JME15 cells.

Figure 4 shows the overall effects of varying concentrations of DSG on human JME/CF15 cells at normal and reduced temperature.

Figure 5 shows the results of SPQ analysis of DSG treated SJBE cells.

Figure 6 shows the overall effects of varying concentrations of DSG on human SJBE cells at normal and reduced temperature.

Figure 7 shows the overall effects of varying concentrations of DSG on a bovine papilloma virus immortalized human airway epithelial cell line containing the ΔF508 variant.

Figure 8 shows the overall effects of varying concentrations of DSG on a pig kidney epithelial cell line containing the ΔF508 variant.

Figures 9A and 9B show the results of SPQ halide efflux assay of IBE-1 cells following treatment with DSG. Figure 9A shows the change in fluorescence in cells treated with DSG, growth at lowered temperature and control. Figure 9B shows the percentage of responsive cells following treatment with different concentrations of DSG or growth at lowered temperature.

Figures 10A-10E show the results of whole cell patch-clamp analysis of IBE-1 cells. Figure 10A shows representative whole cell currents under basal (unstimulated) conditions from IBE-1 cells. Figure 10B shows a recording from the same IBE-1 cells. Figure 10C shows whole cell currents from a cell that had been treated with 10μg/ml DSG for 48 h. Figure 10D shows whole cell currents from the same cell treated with DSG following stimulation with 200μM cpt-cAMP. Figure 10E shows the current-voltage relationships obtained under basal conditions (squares) and after addition of 200μM

cpt-cAMP (circles) of 7 successfully patched cells from 7 different coverslips treated with DSG (10μg/ml) for 48 to 72 h.

Figure 11 shows SPQ analysis of tracheobronchial epithelial cells isolated from ΔF508 mice treated with DSG.

Detailed Description of the Invention

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of conflict or inconsistency, the present description, including definitions, will control.

The language "pharmaceutically acceptable salt" is art-recognized terminology. Typically these salts are capable of being hydrolyzed or solvated under physiological conditions. Examples of such salts include, sodium, potassium, and hemisulfate. The term further is intended to include lower hydrocarbon groups capable of being hydrolyzed or solvated under physiological conditions, i.e. groups which esterify the carboxyl group, e.g. methyl, ethyl, and propyl.

The chaperone modulating agents of the present invention can be purchased or alternatively can be synthesized using conventional techniques.

The language "effective amount" is intended to include that amount sufficient or necessary to

significantly reduce or eliminate a subject's symptoms associated with CF. The amount can be determined based on such factors as the type and severity of symptoms being treated, the weight and/or age of the subject, the previous medical history of the subject, and the selected route for administration of the agent. The determination of appropriate "effective amounts" is within the ordinary skill of the art.

The term administration is intended to include routes of administration which allow the agent (e.g., protein enhancing agent) to perform its intended function, e.g., increasing the level of at least one cellular protein. Examples of routes of administration which can be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, etc.), oral, inhalation, transdermal, and rectal. Depending on the route of administration, the agent can be coated with or in a material to protect it from the natural conditions which may detrimentally effect its ability to perform its intended function. The administration of the agent is done at dosages and for periods of time effective to significantly reduce or eliminate the symptoms associated with CF. Dosage regimes may be adjusted for purposes of improving the therapeutic response of the agent. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The language "CF-associated cell" is intended to include a cell associated with CF which contains normal

and/or mutant CFTR. Examples of such cells include airway epithelial cells such as nasal and lung epithelia.

The present invention further pertains to therapeutic compositions for treating a subject having CF. The composition contains a therapeutically affective amount of a chaperone modulating agent and a pharmaceutically acceptable carrier.

The language "therapeutically effective amount" is that amount sufficient or necessary to significantly reduce or eliminate a subject's symptoms associated with CF. The amount can vary depending on such factors as the severity of the symptoms being treated, the size of the subject, or the selected route for administration of the agent.

The language "pharmaceutically acceptable carrier" is intended to include substances capable of being co-administered with the agent and which allow the agent to perform its intended function, e.g. increasing the intracellular level of at least one cellular protein, inducing differentiation, or protein binding. Examples of such carriers include solvents, dispersion media, delay agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media and agent compatible with the agent can be used with this invention. The agent of this invention can be administered alone or in a pharmaceutically accepted carrier. The agents further can be administrated as a mixture of agents which also can be in a pharmaceutically

acceptable carrier. The agent further can be co-administered with other different art-recognized protein enhancing agents, differentiating agents, and/or adjuvants.

The present invention further pertains to a packaged drug for treating a subject having CF. The packaged drug includes a container holding an agent described above and instructions for administering the agent for treating a subject having CF. Examples of containers include vials, syringes, etc. The instructions would contain dosage information for administering the agent as described above.

One aspect of the instant invention provides that the molecular basis of most cystic fibrosis is the inability of the CFTR gene product to mature. That is to say, the failure of CFTR to move through the normal pathway of intracellular trafficking and modification means that the mature protein is absent from its final cellular destination in CF cells. In normal cells nascent CFTR interacts first with the endoplasmic reticulum and is then glycosylated at least one of Asn residues 894 and 900. The native molecule is then transported to the Golgi where carbohydrate processing to complex-type glycosylation occurs. Finally, at least some of the mature glycosylated molecule is thereafter transported to the plasma membrane.

It is now reasonably well established that the endoplasmic reticulum possesses a mechanism that prevents transport of mutant, misfolded or incorrectly complexed

versions of proteins otherwise destined for further processing (Lodish, 1988; Rose and Doms, 1988; Pelham, 1989; Hurtley and Helenius, 1989; Klausner and Sitia, 1990). If this quality control mechanism operates on CFTR, it would prevent transport to the Golgi and consequently, further modification of several of the mutants reported here. As a result, the unmodified mutant versions of the protein either would not exit the endoplasmic reticulum and would subsequently be degraded therein, or alternatively, they would be transported to the lysosomes for degradation.

It is not clear how the quality control mechanism recognizes the difference between wild-type and those mutant versions of CFTR which were not further processed. One obvious mechanism would be that an alteration in structure of the molecule is detected. Indeed, gross changes in structure of the first nucleotide binding domain (and perhaps in consequence of the whole molecule) might be expected following deletion of phenylalanine 508. However, it is not clear how this change in structure would be detected by a mechanism located, for example, in the lumen of the endoplasmic reticulum, since the domain bearing the mutation, would lie on the cytosolic side of the membrane. Perhaps the structural change is transmitted across the membrane or perhaps the sensing mechanism does not recognize CFTR directly, but rather detects a protein with which it is complexed. In this case, all mutations within CFTR that prevent complex formation also prevent intracellular transport. Yet another mechanism would be that nascent CFTR has basal activity in the endoplasmic reticulum and that mutations

that disrupt this activity are sensed by the quality control mechanism. Perhaps some activity of CFTR is necessary for its maturation and by this means, enzymatically inactive proteins are marked for degradation. Irrespective of the mechanism of discrimination, the time course of synthesis of both wild-type and mutant CFTR is notable in two respects. Firstly, the half life of band B is similar for both wild-type and mutant versions and secondly, most of the wild-type band B appears to be degraded. One interpretation of these results is that synthesis of CFTR involves two steps, retention in the endoplasmic reticulum during which time folding of the protein occurs followed by either export to the Golgi or degradation.

The most common cause of cystic fibrosis is deletion of the phenylalanine residue at position 508 (ΔF508) of the cystic fibrosis transmembrane conductance regulator (CFTR). Studies have shown that this mutation results in the synthesis of a variant CFTR (ΔF508-CFTR) that is defective in its ability to traffic normally to the apical membrane surface where it functions as a chloride (Cl^-) channel (Cheng *et al.* (1990), *Cell* 63:827-834). Rather, most of the nascent ΔF508-CFTR is retained in the endoplasmic reticulum (ER) where it is degraded by a process that involves ubiquitination (Jensen *et al.* (1995), *Cell* 83:129-135; Ward *et al.* (1995), *Cell* 83:121-127). However, functional cAMP-stimulated CFTR Cl^- channel activity can be detected at the plasma membrane when, for example, ΔF508-CFTR is synthesized at a reduced temperature (Denning *et al.* (1992), *Nature* 358:761-764), or in the presence of chemical chaperones (Brown *et al.*

(1996), *Cell Stress & Chaperones* 1:117-125; Sato *et al.* (1996), *J. Biol. Chem.* 271:635-638), and when overexpressed (Cheng *et al.* (1995), *Am. J. Physiol.* 268:L615-L624), indicating that the deletion of phenylalanine 508 does not completely abolish CFTR function. Therefore, strategies that facilitate the relocation or escape of mutant ΔF508-CFTR from the ER to the plasma membrane may be therapeutically beneficial for the treatment of CF.

The mechanisms that result in the retention of the mutant CFTR in the ER have also been studied. The proper folding and assembly of many newly synthesized proteins in the ER is facilitated by molecular chaperones (Hartl, F.U. (1996), *Nature* 381:571-580). These chaperones are thought to promote productive folding in part by preventing aggregation of folding intermediates. One explanation for the retention of CFTR mutations in the ER may be the presence of molecular chaperones in both the ER and the cytosol that prevent newly synthesized proteins from folding inappropriately during processing. Once a protein is correctly folded, it then moves to the Golgi. The immature or band B form of both wild-type CFTR and mutant ΔF508-CFTR have been shown to interact with the ER-resident chaperone calnexin and the cytosolic chaperone hsp70 (Pind *et al.* (1994), *J. Biol. Chem.* 269:12784-12788; Yang *et al.* (1993), *Proc. Natl. Acad. Sci., USA* 90:9480-9484). However, only wild-type CFTR is able to dissociate from either calnexin and hsp70 and exit the ER. In contrast, mutant ΔF508-CFTR is unable to dissociate from either calnexin or hsp70 and does not exit the ER to the Golgi. Both calnexin and hsp70

reportedly retain band B ΔF508-CFTR in the ER and this, it is proposed, contributes to the mislocalization of the mutant CFTR. While interaction of most wild-type CFTR with hsp70 is transient, ΔF508-CFTR forms a stable complex with hsp70 and is degraded in a pre-Golgi nonlysosomal compartment. In ΔF508-CFTR producing cells, only the partially glycosylated band B form, and none of the fully glycosylated band C form of CFTR is generated (Cheng *et al.* (1990), *Cell* 63:827-834). Presumably, the mutant ΔF508-CFTR is recognized as abnormal, perhaps by the chaperones themselves, and is retained in the ER where it is subsequently degraded. The finding that hsp70 and calnexin may be responsible for the ER retention of ΔF508-CFTR raises the possibility of therapeutic intervention in CF by agents capable of interfering with the normal functioning of these chaperones.

Given that ΔF508-CFTR has been shown to be functionally competent when it is able to reach the plasma membrane, methods and compositions which promote trafficking of this mutant to the plasma membrane provide the basis of novel approaches to CF therapy. Accordingly, the present invention provides methods and compositions capable of disrupting the CFTR-molecular chaperone complex. For example, drugs active in altering the activity and distribution of hsp70 or calnexin proteins could advantageously be used to redistribute to the plasma membrane mutant CFTR which retains at least some functional activity. Similarly, agents effective in stimulating sufficient CFTR activity to result in export of otherwise mutant CFTR to the Golgi for additional

glycosylation could result in improved CFTR function in homozygous CF individuals. Alternatively, therapeutic treatment via a suitable, therapeutically effective blocking agents could be used to deactivate chaperone proteins, for example, agents that are substrates and compete for binding to hsp70 or calnexin.

Examples of agents that bind to hsp70, include, but are not limited to deoxyspergualin (DSG), a spermidinyl, α-hydroxyglycyl, 7-guanidinoheptanoyl peptidomimetic, and analogs thereof, for example, methoxy- and glycylDSG have been shown to bind hsps with similar affinities (Nadler *et al.* (1992), *Science* 258:484-486). Pure human hsp90 and hsp70 have equivalent affinities for DSG. Hsp90 is particularly abundant cytosolic protein and its concentration may approach 2-10 μM, while hsp70 can reach 5μM. Given kds of 4-5μM, the DSG-hsp complexes would be highly populated and DSG could compete effectively for other protein and peptide binding to hsp70 and hsp90 and thereby affect protein trafficking (Nadeau *et al.* (1994), *Biochemistry* 33:2561-2567).

DSG, a potent immunosuppressive agent, is a stable synthetic analogue of a natural product, spergualin, originally isolated from *Bacillus laterosporus* (Umezawa *et al.* (1981), *J. Antibiotics* 34:1622-1624). DSG has demonstrated potent immunosuppressive activity in a number of T-cell dependent assays and models. It has been suggested that this immunosuppressive activity is mediated, at least in part, through its ability to interact with hsc70 and hsp90 (Nadler *et al., supra*; Nadeau *et al., supra*.). DSG has the unique ability to

suppress both humoral and cell-mediated immune responses by down-regulating presentation of MHC class I or II antigen, modulating IL-1 production, and inhibiting IL-2 receptor expression. Perhaps most importantly it also has the effect of preventing monocytes from functioning as antigen-presenting cells.

It has been suggested that DSG works by blocking hsp70's ability to transport proteins, specifically NF-κß into the nucleus (Tepper *et al.* (1995), *J. Immunol.* 155:2427-2436). The working hypothesis is that DSG binds to hsc70, the constitutively expressed form of the hsp 70 family, that normally serves to fold and chaperone proteins across membranes. It appears that DSG is binding at a site normally occupied by the hsp helper protein dna J, interfering with ATPase activity in a still undetermined way (Nadler *et al.* (1995) *Therapeutic Drug Monitoring* 17:700-703).

Accordingly, the present invention provides novel methods and compositions for treating cystic fibrosis-associated (CF-associated) cells with agents that interfere with and/or modulate the functioning of molecular chaperone proteins thereby allowing the mutant CFTR protein to escape from the ER, proceed to the plasma membrane and provide functional cAMP-responsive Cl^- channels.

In tests to ascertain whether binding of agents to the chaperones is sufficient to alter the trafficking and, hence, the subcellular location of ΔF508-CFTR, cells expressing the mutant protein were exposed to DSG.

Results of experiments described herein show that addition of DSG to cells expressing recombinant ΔF508-CFTR resulted in the appearance of functional cAMP-stimulated CFTR Cl^- channel activity at the cell surface. Moreover, DSG also restored cAMP-mediated CFTR Cl^- channel activity in human CF airway and biliary epithelial cells.

Discussion

Several therapeutic approaches are being developed concurrently for the treatment of CF. These include (i) agents that improve the anti-bacterial activity and viscosity of the mucus fluids lining the airways, (ii) agents that by-pass the CFTR Cl^- channel defect, (iii) protein and gene augmentation therapy, and (iv) agents that reverse the mutant phenotype. Examples of the last group include aminoglycosides to suppress disease-associated stop mutations (Howard *et al.*(1996), *Nature Genetics* 2:467-469) and phenylbutyrate (Cheng *et al.*(1995), *supra;* Rubenstein *et al.* (1996), *Pediat. Pulmonology* 13:259) and chemical chaperones (Brown *et al.*, *supra.*; Sato *et al.*, *supra.*) to reverse trafficking mutants.

The trafficking, or Class II-type mutations as exemplified by ΔF508, are the most common among CF patients. The variant ΔF508-CFTR is recognized as abnormal and purportedly retained by the molecular chaperones hsp70 and calnexin in the ER where it is subsequently degraded (Yang *et al.*(1993), *Proc. Natl. Acad. Sci., USA* 90:9480-9484; Pind *et al.* (1994), *J.*

Biol. Chem. 269:12784-12788). A premise of the present invention is, therefore, that agents capable of disrupting the interaction of ΔF508-CFTR with its molecular chaperones might facilitate escape of the variant protein from the quality control apparatus in the ER and, thereby, allow transit to the plasma membrane.

Deoxyspergualin, an immunosuppressant presently under clinical investigation, binds hsc70 and hsp90 with affinities that are predicted to complete effectively for the binding of these chaperones to nascent polypeptides (Nadeau *et al., supra.*). It is reported herein that DSG is indeed capable of partially reversing the trafficking defect associated with ΔF508-CFTR in recombinant and immortalized human CF epithelial cell lines.

ΔF508-CFTR cells exposed to DSG exhibited cAMP-stimulated Cl^- channel activity, a function that was otherwise lacking in these cells. These results are interpreted to mean that DSG was able to salvage a fraction of the mutant CFTR normally targeted for degradation by hsp70 and calnexin and, thereby, allowed for the translocation of at least some ΔF508-CFTR to the plasma membrane.

Although functional cAMP-stimulated Cl^- channels were detected in a proportion of the DSG-treated cells, the presence of any mature band C-form of CFTR could not be demonstrated by immunoprecipitation analysis. However, patch-clamp experiments clearly demonstrated the presence of functional CFTR at the plasma membrane in cells treated with DSG. This result would argue that

either a very small amount of ΔF508-CFTR that was below the level of detection using the biochemical assays escaped the ER to the Golgi and thence to the plasma membrane, or that the form that trafficked to the plasma membrane was indistinguishable from the core-glycosylated band B form. Although it is not unreasonable to speculate that DSG affected the trafficking and thereby the subcellular location of ΔF508-CFTR by altering its relationship with molecular chaperones, because of the inability to detect band C-CFTR, other mechanisms cannot be excluded.

The response observed with DSG was also compared with other interventions shown previously to result in the presence of ΔF508-CFTR at the plasma membrane. In both the CF airway and biliary epithelial cell lines, the response attained with DSG was comparable to that observed when these cells were cultured at a reduced temperature. In recombinant cells, the effect of incubation at low temperature has been shown to be as effective as treatment with the chemical chaperone glycerol in eliciting the presence of ΔF508-CFTR at the cell surface (Brown *et al.*, *supra.*; Sato *et al.*, *supra.*). In this regard, DSG would appear to be as effective as any other treatment shown previously to be capable of rescuing the ΔF508-CFTR trafficking defect.

If the mechanism by which DSG effected the presence of ΔF508-CFTR at the plasma membrane was indeed mediated through its interaction with the chaperones that normally associate with ΔF508-CFTR, then other interventions aimed at eliciting a similar release of the chaperones from the

newly synthesized mutant CFTR might induce a portion of the protein to undergo maturation and transit to the cell surface. For example, heat shock treatment, which results in a rapid redistribution of hsp70 from the cytoplasm to the nucleus, might also result in the release of a small proportion of the mutant CFTR. Immunosuppressive Allotrap peptides derived from highly conserved regions of human MHC Class I molecules are capable of binding hsp70 and may also be similarly efficacious (Nossner *et al.* (1996), *J. Exp. Med.*183:339-348). However all of these interventions are non-specific, and as such are likely to result in a general disruption of the quality control apparatus that normally regulates proper folding and trafficking of proteins in the cell. It is unclear whether such a general disruption would adversely affect long term cell viability. Nevertheless, the results reported herein suggest that identification of agents like DSG that perhaps are more specific for ΔF508-CFTR or which act only transiently may be efficacious for the treatment of CF. Furthermore, because the mechanisms of action of sodium butyrate and the chemical chaperones are different from that of DSG, the use of a combination of these agents may be synergistic and result in even greater levels of ΔF508-CFTR at the plasma membrane. Finally, one may also consider inclusion of compounds like genistein and calyculin shown recently to enhance the activity of CFTR chloride channels at the cell surface (Yang *et al.* (1997), *Am. J. Physiol.* 272:C142-C155).

The following examples are intended to illustrate the invention without limiting the scope thereof.

Experimental Protocols

Assessment of CFTR functional activity using fluorescence digital imaging microscopy.

The cAMP-dependent CFTR Cl^- channel activity was assessed using the halide-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ) essentially as described previously (Cheng *et al.*, (1995), *supra.*; Marshall *et al.*, *supra.*). Briefly, the cells were treated with different amounts of DSG for the times specified. At the end of the treatment period, the cells were loaded with SPQ by hypotonic shock for 4 min at room temperature. SPQ fluorescence initially was quenched by incubating the cells for up to 30 min in a NaI buffer (135mM NaI, 2.4mM K_2HPO_4, 0.6mM KH_2PO_4, 1mM $MgSO_4$, 1mM $CaSO_4$, 10mM dextrose, and 10mM N-2 hydroxyethylpiperazine-N'2-ethanesulfonic acid, pH 7.4). After measuring the baseline fluorescence (Fo) for 2 min., the NaI solution was replaced with one containing 135mM $NaNO_3$, and fluorescence was measured for another 16 min. Forskolin (20μM) and 3-isobutyl-1-methyl-xanthine (IBMX) (100μM) were added 5 min after the anion substitution to increase intracellular levels of cAMP.

An increase in halide permeability is reflected by a more rapid increase in SPQ fluorescence. It is the rate of change rather than the absolute change in signal that is the important variable in evaluating anion permeability. Differences in absolute levels reflect quantitative differences between groups in SPQ loading, size of cells, or number of cells studied. The data are

presented as means ± SE of fluorescence at time t (Ft) minus the baseline fluorescence (Fo, the average fluorescence measured in the presence of I^- for 2 min. before ion substitution) and are representative of results obtained under each condition.

For each experiment, between 50 to 100 cells were examined on a given day and studies under each condition were repeated on at least two days. For each experiment, the responses were compared with those obtained with control or untreated cells. Cells were scored as positive if they exhibited a rate of change in fluorescence that was greater than the signal observed with the control cells. Under the conditions specified above, control cells were unresponsive to added cAMP agonists. There was a broad spectrum in the rate of change in SPQ fluorescence observed with responsive cells. Normally, cells were scored as responsive if they generated a minimum of a 20° change in the rate of increase in SPQ fluorescence following stimulation with cAMP agonists. Because the response was heterogenous, the data shown are for the 10% of cells in each experiment showing the greatest response.

Whole cell patch-clamp recording.

Whole cell patch-clamp recordings were performed essentially as described previously (Hamill *et al.*, (1981), *Pflügers Arch.* 391:85-100; Egan *et al.* (1992), *Nature* 358:581-584; Anderson *et al.* (1991), *supra.*; Sheppard *et al.*, (1996). Briefly, cells on coverslips were placed in a chamber mounted on a Nikon Diaphot

inverted microscope. Patch pipettes had resistances of 2-4 MΩ. Whole cell configuration was achieved with an additional pulse suction to rupture the gigaseal. The pipette (intracellular) solution contained: 130mM CsCl, 20mM TEA-Cl, 10mM N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10mM ethylene glycol-bis-(β-aminoethylether) N, N, N', N'-tetraacetic acid (EGTA), 10mM Mg-ATP, and 0.1mM LiGTP, pH 7.4. The bath (extracellular) solution contained: 140mM N-methyl-D-glutamine (NMDG), 2mM $CaCl_2$, 1mM $MgCl_2$, 0.1mM $CdCl_2$, 10mM HEPES, 4mM CsCl, and 10mM glucose, pH 7.4. These solutions were designed so as to study only currents flowing through Cl^- channels, since Cl^- was the only significant permeant ion in the solutions. Furthermore, Ca^{2+}-activated Cl^- currents were minimized by inclusion of 10mM EGTA in the intracellular solution and 100 μM of Cd^{2+} in the extracellular bath. K^+ currents were minimized by including 20mM TEA in the intracellular solution. Aspartate was used as the replacement anion in experiments in which extracellular Cl^- concentration was changed. Forskolin (10μM), IBMX (100μM), 8-(4-chlorophenylthio)-cAMP (cpt-cAMP, 200μM), diphenylamine carboxylic acid (DPC, 200μM), UTP (100μM), and ionomycin (1μM) were added to the bath solutions as indicated. Current recordings were made from the same cells before, during, and after exposure to the solutions containing the different agonists or inhibitors. All experiments were performed at room temperature (22°C). Currents were filtered at 2kHz. Data acquisition and analysis were performed using the pCLAMP 5.5.1 software (Axon Instruments, Foster City, CA).

Biochemical analysis of CFTR.

Detailed procedures for preparing cell lysates, immunoprecipitation, phosphorylation of CFTR using protein kinase A and [γ^{32}P]ATP, and polyacrylamide gel electrophoresis have all been described previously (Cheng *et al.*, (1995), *supra.*; Marshall *et al.*, *supra.*).

Example 1 - The Effect of DSG on Recombinant C127 Cells Expressing ΔF508 Cystic Fibrosis Transmembrane Regulator Protein

Nadler *et al.* (1992), *Science* 258:484-486 has reported that the immunosuppressant deoxyspergualin (DSG) interacts with hsc70, a member of the hsp70 family of heat shock proteins. The K_d value for DSG binding to hsc70 is 4μM, a concentration that is within the range of a pharmacologically active dose (Nadeau *et al.* (1994), *Biochemistry* 33:2561-2567). Since the intracellular concentration of hsc70 is approximately 5μM, it is possible that DSG binding to hsp70 may compete effectively for peptide or protein binding to hsp 70.

Derivation of ΔF508-C127 Cells

A bovine-papilloma virus based eukaryotic expression vector (pBPV-CFTR-ΔF508) containing the gene for ΔF508 CFTR and neomycin resistance were transfected into C127 cells. The C127 cells are murine mammary cells which were obtained from ATCC (#CRL 1616). The expression of the mutant ΔF508 protein and neomycin was driven using a metallothionein promoter. Following transfection, clonal

cells resistant to G418 were isolated and cells expressing the mutant ΔF508 protein were subsequently identified using antibodies specific for CFTR (mAb-13-1). The cells expressing the mutant ΔF508 CFTR protein were maintained in Dulbecco's modified eagle media (DMEM) supplemented with glutamine and fetal calf serum.

C127-ΔF508-low (mouse mammary tumor) is a recombinant cell line stably transfected with the cDNA encoding the mutant ΔF508-CFTR (Cheng *et al.* (1995), *supra.*; Marshall *et al.* (1994), *J. Biol. Chem.* 269:2987-2995). These cells produce solely the immature, partially glycosylated band B form of CFTR (characteristic of processing only in the ER) and do not exhibit detectable CFTR Cl^- channel activity at the cell surface (Cheng *et al.* (1995), *supra.*). C127-mock is a cell line that has been stably transfected with the backbone of the expression vector used to generate C127-ΔF508-low (Cheng *et al.* (1995), *supra.*). $LLCPK_1$-ΔF508 (pig kidney epithelial) is a recombinant cell line stably expressing low levels of the mutant ΔF508-CFTR protein (Marshall *et al.*, *supra.*). The details of the generation, characterization and routine propagation of these cell lines has been described (Cheng *et al.* (1995), *supra.*; Marshall *et al.*, *supra.*).

Cells were treated with up to 100μg/ml of DSG for up to 72 h. Concentrations of DSG >50μg/ml were toxic to most of the cell types tested. Since DSG is modified by polyamine oxidase present in fetal bovine serum (Tepper *et al.*, *supra.*), cells were routinely replenished with fresh medium containing DSG and aminoguanidine every 24

h. As a control in some experiments, C127 cells were also treated with 5mM sodium butyrate for 24 h. to enhance expression of ΔF508-CFTR (Cheng *et al.* (1995), *supra.*). As another control, cells were cultured at 23°C for 24 to 48 h. to facilitate folding of the mutant, ΔF508-CFTR at the ER.

Treatment of the ΔF508-C127 Cells with DSG and Analysis of Cells for Chloride Channel Activity

To test whether treatment with DSG may interfere with the ability of hsp70 to retain ΔF508-CFTR in the ER, recombinant C127 cells expressing ΔF508-CFTR were seeded onto glass coverslips and were exposed to DSG (10 to 100 μg/ml) (Bristol Myers Squibb, Seattle, WA) for 48 to 72 h. Analysis for evidence of mature band C-CFTR (Cheng *et al.* (1990), *supra.*) which would be indicative of processing of ΔF508-CFTR in the Golgi was performed. Biochemical analysis of the lysates from these cells showed no discernible evidence of the mature band C form of CFTR indicating that very little if any ΔF508-CFTR had exited the ER to the Golgi.

Immunoprecipitation analysis of C127-ΔF508-low cells

Lysates were prepared from C127 cells stably expressing ΔF508-CFTR (Fig. 1A, lanes 1-3) or wild-type CFTR (lane 4). Cells were treated with either 10μg/ml DSG (lane 3) or left untreated (lanes 1 and 4) for 72 h before lysis. Immunoprecipitates obtained using the anti-CFTR monoclonal antibody mAB 24-1 (Marshall *et al.*, *supra.*) were phosphorylated *in vitro* by the addition of

the catalytic subunit of protein kinase A and [γ^{32}P]ATP. The positions of band B (core-glycosylated CFTR) and band C (mature form of CFTR) are indicated on the right.

Example 2 - SPQ Analysis of C127-ΔF508-low Cells Following Treatment with DSG

To ascertain whether a small amount of ΔF508-CFTR, below the sensitivity of detection with the biochemical assay, may have traversed the Golgi to the plasma membrane the more sensitive single-cell membrane halide permeability assay using the Cl^- indicator SPQ (6-methoxy-N-(sulfopropyl)-quinolinium) (Cheng *et al.* (1995), *supra.*; Marshall *et al.*, *supra.*; Cheng *et al.* (1991) *Cell* 66:1027-1036). Accordingly, following treatment with DSG, the cells were assayed for the presence of functional CFTR chloride channel activity at the cell surface using the halide sensitive fluorophore SPQ assay. In this assay, a rapid change in SPQ fluorescence upon stimulation with cAMP agonists is indicative of the presence of active CFTR at the plasma membrane.

Cells were loaded with SPQ by either including 10mM SPQ (Molecular Probes, Eugene, OR) in the growth media for nine to twelve (or twelve to eighteen) hours or after hypotonic shock (with 50% vol/vol water) for 4 min at room temperature. SPQ fluorescence was initially quenched by incubating the cells for up to 30 min. in a sodium iodide buffer solution (135mM NaI; 2.4mM K_2HPO_4; 0.6mM KH_2PO_4; 1.0mM $MgSO_4$; 1.0mM $CaSO_4$; 10mM dextrose and 10.0mM HEPES pH 7.4). After measuring the baseline

fluorescence for two minutes using a Nikon inverted microscope, a Universal Imaging System and a Hamatsu camera, the sodium iodide buffer solution was replaced by a sodium nitrate buffer solution (same as the NaI solution except $NaNO_3$ was substituted for NaI) at time = 0 min. and fluorescence was measured for an additional 16 minutes. SPQ fluorescence is quenched by iodide but not by nitrate. Intracellular cAMP levels were increased by adding forskolin (Calbiochem, San Diego, CA) and 3-isobutyl-1-methyl-xanthene (IBMX) (Sigma, St. Louis, MO) after the anion substitution at time = 4 min. In this assay (hereinafter the SPQ assay) an increase in halide permeability results in SPQ fluorescence.

In this assay, a rapid change in fluorescence upon stimulation with cAMP agonists (i.e., forskolin) is indicative of the presence of active CFTR at the plasma membrane. Fig. 1B shows that DSG-treated C127-ΔF508-CFTR cells gave a rapid change in fluorescence following stimulation with forskolin indicating that incubation with DSG effected the presence of functional CFTR activity at the cell surface. These cells, therefore, contained functional cAMP-dependent chloride channels. This activity was absent from ΔF508-C127 cells which had not been pretreated with DSG, but had been mock treated with phosphate buffered saline.

Example 3 - The Effect of DSG Concentration on Recombinant C127 Cells Expressing ΔF508 Cystic Fibrosis Transmembrane Regulator Protein

Results similar to those in Example 2 were obtained in another experiment (Figs. 1C and 1D) using different concentrations of DSG (10 μg/ml; 20 μg/ml; 50 μg/ml). More optimal responses were observed in cells that were treated with lower concentrations of DSG compared to higher concentrations of DSG. However, this difference could be attributed to cytotoxicity associated with higher concentrations of DSG. The results of the study, which are presented in Fig.1E, also indicate that percentage of mature CFTR produced from CFTR-ΔF508 in the presence of DSG increases upon exposure to reduced temperatures.

In Fig. 1C, change in fluorescence is shown for C127 cells expressing ΔF508-CFTR (n=9, where n=number of cells), cells expressing ΔF508-CFTR that had been treated with 5mM sodium butyrate (Sigma) for 24 h. (n=11), or 10μg/ml DSG (Bristol Myers Squibb) for 72 h (n=10), or incubated at 23°C for 24 h. (n=12), and mock transfected C127 cells that had been similarly pre-treated with DSG (n=7). Data are presented as the fluorescence at time **t** (Ft) minus the baseline fluorescence (Fo, average fluorescence measured in the presence of I^- for 2 min. before ion substitution). Data are means ± SEM and are representative of responses obtained from several experiments for each condition.

As previously reported (Cheng *et al.* (1995), *supra.*) either treating the ΔF508-CFTR-low cells with sodium butyrate to augment the expression of ΔF508-CFTR or culturing them at a reduced temperature (23°C) to enhance folding (Denning *et al.*, *supra.*) generated cAMP-stimulated halide efflux. Cells that were grown in the presence of DSG for 3 days also restored cAMP-stimulated anion efflux albeit to a lesser extent than was observed with sodium butyrate treatment or following a temperature shift. Approximately 17% of the DSG-treated C127-ΔF508-CFTR-low cells generated a measurable response as compared to 90% with sodium butyrate treatment or following growth at low temperature (average of 5 experiments). This disparity in response was not unexpected since treatment of these cells with sodium butyrate or growth at reduced temperature, unlike treatment with DSG, results in synthesis of detectable amounts of band C-CFTR (Denning *et al.*, *supra.*; Cheng *et al.* (1995); *supra.*). Exposure to higher concentrations of DSG (>50μg/ml) was toxic to the cells and did not improve either the intensity or frequency of the signal.(Figs. 1D and 1E) No response was observed in C127-ΔF508-low cells that were left untreated or in C127-mock cells (parental C127 cells mock transfected with expression vector alone) that had been treated with DSG (Fig. 1C). These results suggest that the Cl^- channels observed in the DSG-treated C127-ΔF508-low cells were most likely due to the presence of mutant ΔF508-CFTR at the cell surface.

Since the structure of DSG resembles that of the polyamine spermidine, C127-ΔF508-low cells were also

treated with 5μg/ml spermidine for 72 h. as a negative control. No measurable cAMP-stimulated Cl^- channel activity was detected following treatment with spermidine, arguing against a non-specific effect. Data similar to those described for C127-ΔF508-low cells were also observed with $LLCPK_1$-ΔF508 cells, a recombinant pig kidney epithelial cell line stably expressing the variant CFTR.

Under the conditions tested, the change in SPQ fluorescence observed in DSG-treated cells was less than that obtained with sodium butyrate, an agent described previously as capable of effecting the presence of ΔF508-CFTR at the plasma membrane of these cells (Cheng *et al.* (1995), *Am. J. Physiol.* 268:L615-L624). It is possible that with optimization of the dose of DSG, and addition of the polyamine oxidase inhibitor aminoguanidine hemisulfate to prevent the breakdown of DSG (Tepper *et al.* (1995), *J. Immunol.* 155:2427-2436), greater responses may be realized. Furthermore, because the mechanism of action of butyrate and DSG are likely to be different, use of both agents together may be synergistic and result in even greater levels of ΔF508-CFTR at the plasma membrane.

Example 4 - The Effect of DSG on Immortalized Human Airway Epithelial Cells Expressing ΔF508 Cystic Fibrosis Transmembrane Regulator Protein

Cell Culture

CFT1 and JME/CF15 are two immortalized human CF airway epithelial cell lines which contain the ΔF508 variant (Jefferson *et al.* (1990), *Am. J. Physiol.* 259:L496-L505; Yankaskas *et al.*, (1993), *Am. J. Physiol.* 264:C1219-C1230). Cells were treated with up to 100μg/ml of DSG for up to 72 h. Concentrations of DSG >50μg/ml were toxic to most of the cell types tested. Since DSG is modified by polyamine oxidase present in fetal bovine serum (Tepper *et al., supra.*), cells were routinely replenished with fresh medium containing DSG and aminoguanidine every 24 h. As a control in some experiments, cells were cultured at 23°C for 24 to 48 h. to facilitate folding of the mutant ΔF508-CFTR at the ER.

Immortalized CF nasal airway epithelial cells (JME/CF15) generated from a ΔF508 (-/-) patient, were maintained by coculturing with lethally irradiated NIH-3T3 cells in Dulbecco's modified eagles medium/F-12 (3:1) supplemented with adenine, insulin, transferrin, triiodothyronine, hydrocortisone, cholera toxin, epidermal growth factor and 5% fetal bovine serum. Before use cells were grown to confluence to eliminate all cocultured NIH-3T3 cells.

Treatment of the JME/CF15 (or CFT1) Cells with DSG and Analysis of Cells for Chloride Channel Activity

To test whether treatment with DSG had a similar effect on human CF cells, an immortalized airway epithelial cell line (JME/CF15) obtained from a CF patient homozygous for the ΔF508 mutation was treated with DSG. Attempts to detect changes in the glycosylation state of CFTR following treatment with DSG or sodium butyrate or growth at reduced temperature by immunoprecipitation assays were unsuccessful due to the low amounts of CFTR in these cells. This was not surprising since many similar labeling experiments in the past using primary normal human airway epithelial cells also failed to detect CFTR due to its low abundance. Examination of the untreated JME/CF15 cells using the SPQ assay showed, as expected, a lack of detectable cAMP-stimulated Cl^- channel activity (Fig.2A).

Additionally, consistent with expectations, when these cells were grown at 23°C for 24 h., measurable cAMP-regulated Cl^- channel activity could be detected in a proportion of the cells. Cells pre-treated with between 10 and 100μg/ml DSG for 72 h. also displayed cAMP-responsive Cl^- channel activity. The effect was specific for DSG and was not replicated with the structurally related analogue spermidine.

The response observed with DSG appeared more robust than that attained when cells were cultured at low temperature. For example, the cAMP-stimulated rate of change in SPQ fluorescence observed with DSG was

consistently greater (Fig.2A) and the total number of responsive cells (approximately 10-15%) was slightly higher (Fig. 2B) than that observed when the cells were cultured at low temperature. This is contrary to what was observed with the recombinant C127-ΔF508-low cells. However, it should be noted that DSG also has an ascribed role in blocking the nuclear translocation of the transcriptional factor NF-κB (Tepper *et al., supra.*). This block may have reduced the transcriptional activity of the CMV promoter (which contains several consensus NF-κB binding sites) used to express ΔF508-CFTR in the C127 cells and, therefore, reduced the levels of mutant protein produced in these cells. Although the percentage of responsive cells observed with DSG was only approximately 12% (Fig. 2B), it should be noted that this determination was limited by the sensitivity of the SPQ assay and that the number of cells affected may be greater.

Although a greater number of responsive cells was observed when 10μg/ml of DSG was used instead of 5μg/ml, no further significant increment in response was noted at concentrations higher than 10μg/ml. It appears, therefore, that DSG would be capable of generating functional cAMP-stimulated Cl^- channel activity in at least a proportion of the immortalized ΔF508 human airway epithelial cells. Because many studies have indicated that these cells lack cAMP-dependent Cl^- channel activity other than CFTR (Jefferson *et al., supra.*), the observed response after DSG treatment was most likely due to ΔF508-CFTR at the cell surface. The above experiments have been repeated using another immortalized human CF

airway epithelial cell line, CFT1, with very similar results.

To test whether treatment with DSG may interfere with the ability of hsp70 to retain ΔF508-CFTR in the ER, JME/CF15 (or CFT1) cells expressing ΔF508 CFTR were seeded onto glass coverslips and were exposed to DSG (10 μg/ml; 50 μg/ml; 100 μg/ml) (Bristol Myers Squibb, Seattle, WA) for 48 to 72 h.

Following treatment with DSG, the cells were assayed for the presence of functional CFTR chloride channel activity at the cell surface using the halide sensitive fluorophore 6-methoxy-N-[3-sulfopropyl]-quinolinium (SPQ) assay (Cheng *et al.* (1991) *Cell* 66:1027-1036). See, also Example 2 above.

Fig. 3 shows that DSG at doses of 10, 50, and 100 μg/ml increased cAMP-mediated chloride channel activity in immortalized airway epithelial (JME/CF15) cells generated from a ΔF508 (-/-) patient measured by SPQ. The results of the study, which are presented in Fig. 4, indicate that percentage of mature CFTR produced from CFTRΔF508 in the presence of DSG increases upon exposure to reduced temperatures.

Similar results were obtained in an SV40 immortalized human intrahepatic biliary duct epithelial (SJBE) cell line containing the ΔF508 variant (Figs. 5 and 6). However, in cell lines expressing recombinant ΔF508 under the control of a CMV promoter, namely C127 cells and $LLCPK_1$ cells, the effects of DSG on

cAMP-mediated chloride channel activity measured by SPQ was marginal (Figs. 7 and 8).

These results suggest that DSG may rescue ΔF508 CFTR by affecting its trafficking in immortalized human CF epithelial cells. The mechanism, possibly through an interaction with hsp 70, is different from that of sodium butyrate which has been shown to cause overexpression of ΔF508 in recombinant cells.

Example 5 - The Effect of DSG on Primary Airway Epithelial Cells Expressing ΔF508 Cystic Fibrosis Transmembrane Regulator Protein

To test whether treatment with DSG may interfere with the ability of hsp70 to retain ΔF508-CFTR in the ER, primary tracheobronchial epithelial cells from transgenic ΔF508 (-/-) mice are seeded onto glass coverslips and exposed to varying concentrations of DSG (10μg/ml to 100 μg/ml) (Bristol Myers Squibb, Seattle, WA) for a period of 3 - 5 days.

Following treatment with DSG, the cells are assayed for the presence of functional CFTR chloride channel activity at the cell surface using the halide sensitive fluorophore 6-methoxy-N-[3-sulfopropyl]-quinolinium (SPQ) assay (Cheng *et al.* (1991) *Cell* 66:1027-1036). See, also Example 2 above.

Example 6 - The Effect of DSG on Immortalized CF Biliary Epithelial Cells

Cell Culture

IBE-1 is an immortalized human CF intrahepatic biliary epithelial cell line which contain the ΔF508 variant and G542X mutation (premature stop mutation at residue 542) (Grubman *et al.* (1995), *Gastroenterology* 108:584-592). Cells were treated with up to 100μg/ml of DSG for up to 72 h. Concentrations of DSG >50μg/ml were toxic to most of the cell types tested. Since DSG is modified by polyamine oxidase present in fetal bovine serum (Tepper *et al.*, *supra.*), cells were routinely replenished with fresh medium containing DSG and aminoguanidine every 24 h. As a control in some experiments, cells were cultured at 23°C for 24 to 48 h. to facilitate folding of the mutant ΔF508-CFTR at the ER.

Treatment of the IBE-1 Cells with DSG and Analysis of Cells for Chloride Channel Activity

The ability of DSG to influence the presence of endogenous mutant ΔF508-CFTR at the plasma membrane cAMP-stimulated Cl^- channel activity also assessed in IBE-1 cells. Consistent with previous reports (Grubman *et al.*, *supra.*), IBE-1 cells did not exhibit any measurable cAMP-stimulated Cl^- channel activity (Fig. 9A). However, upon exposure to between 5 and 50μg/ml DSG for 72 h., up to 20% of the cells exhibited measurable cAMP-stimulated Cl^- channel activity (Fig. 9B). The effect of DSG on IBE-1 cells was concentration-dependent, with higher

concentrations of DSG giving rise to greater transport rates and higher numbers of positively-responding cells. Moreover, the response observed at the higher concentrations was similar to that attained when these cells were cultured at reduced temperature.

Whole Cell Patch-Clamp Analysis of IBE-1 Cells Treated with DSG

Whole cell patch experiments were performed to further demonstrate the presence of functional CFTR across the cytoplasm in cells treated with DSG. In all of the successfully patched CF IBE-1 cells from 6 separate coverslips no activation of whole cell currents in response to forskolin (10μM) plus IBMX (100μM) or cpt-cAMP (200μM) was detected (Fig. 10A and 10B), indicating no endogenous CFTR channel activity in these cells. In contrast, in 7 out of 19 successful patched cells from 19 separate coverslips treated with DSG (10μM) for 48 to 72 hrs. a significant activation of whole cell currents was observed. One of the representative experiments is shown in Fig. 10C and 10D. As all the other cells, the holding potential was 0mV, which inactivated the voltage-gated Na^+ and Ca^{++} channels. After addition of 200μM cpt-cAMP to the bath solution, voltage steps from the holding potential to between -100mV and 80mV in 20mV increments activated whole cell currents. In these experiments, the intracellular and extracellular solutions were designed to study only current flowing through Cl^- channels, since Cl^- is the only significant permeant ion in solution. Ca^{++} and K^+ currents were minimized by 100μM of extracellular Cd^{++} and 20mM of intracellular TEA

respectively. Furthermore, the currents were ascertained to be Cl^- currents from change in the reversal potential which was shifted from 1.1 ± 1.5 to 47.3 ± 6.1 mV by reducing extracellular Cl^- from 150mM to 20mM. The current/voltage relationships are summarized in Fig. 10E. The successfully patched cells had whole cell properties that resembled those of wild-type CFTR in epithelial cells (Welsh *et al.* (1995), Cystic Fibrosis in *THE METABOLIC BASIS OF INHERITED DISEASE* (7th ed.) (Scriver, Beaudet, Sly and Valle, eds.) pp. 3799-3876, McGraw-Hill, New York; Riordan, J.R. (1993), *Annu. Rev. Physiol.* 55:609-630).

It is highly unlikely that Ca^{++}-activated Cl^- was recorded in these experiments because of the presence of 10mM intracellular EGTA and 100μM extracellular Cd^{++}. Under these experimental conditions, 100μM UTP plus 1μM ionomycin failed to activate whole cell currents. Special attention was paid to detect possible contribution of the outwardly rectifying Cl^- channel (ORCC) to the whole cell currents. Because of a possible upregulatory role of CFTR, ORCC can be activated in normal but not in CF airway epithelial cells (Hwang *et al.* (1989), *supra.*; Li *et al.* (1988), *Nature* 331:358-360; Morris and Frizzel (1993); *Am. J. Physiol.*; Schwiebert *et al.* (1994), *Am. J. Physiol.*). Any involvement of ORCC may provide evidence that DSG restored CFTR function which in return upregulated ORCC. However, several laboratories found that cAMP-stimulated whole cell currents had properties most consistent with activation of CFTR and ORCC did not contribute to cAMP-activated whole cell currents (Bear and Reyes (1992), *Am. J.*

Physiol.; Gray *et al.* (1993); Haws *et al.* (1992); Wagner *et al.* (1991), *Nature*; Cliff and Frizzel (1990), *Proc. Natl. Acad. Sci., USA*).

In the whole cell patch studies described herein, there is evidence suggesting that the contribution of ORCC to the whole cell currents in these experiments was minimal, if any. Firstly, all experiments were performed at room temperature. ORCC is rapidly inactivated at room temperature and the remaining current is CFTR (Schwiebert *et al., supra*). Secondly, recombinant human CFTR was transduced into CF IBE-1 cells using an adenovirus vector expressing CFTR (Jiang *et al.* (1996), *Am. J. Physiol.* L527-L537). Whole cell currents recorded in CF IBE-1 cells treated with DSG were almost identical to that recorded in transduced CF IBE-1 cells. Finally, the whole cell currents were markedly (92 ± 4%, n=4) reduced by 200μM of extracellular DPC which has been shown to specifically inhibit CFTR but not ORCC (Cliff and Frizzel, *supra.*; Schwiebert *et al., supra.*)

Example 7 - *In vivo* DSG experiments

The transgenic ΔF508 mouse was generated by introducing the ΔF508 gene using a replacement-type targeting construct (Colledge, W.H. *et al.*(1995), *Nature Genetics* 10:445-452). The CF mice, bred at Genzyme Corporation (Framingham, MA), were subcutaneously injected with either DSG (10mg/kg/day in 100μl PBS) or PBS (100μl PBS) for 5 days prior to sacrifice. The airway epithelial cells were enzymatically isolated within 4 to 6 hours and then plated on collagen-coated

glass cover-slips. SPQ analysis was performed 36 to 48 hours later. Fig. 11 shows that a cocktail of forskolin (10μM) and IBMX (100μM) stimulated a rapid increase in SPQ fluorescence in cells isolated from mice treated with DSG. In contrast, no significant increase in SPQ fluorescence in response to the cAMP agonists was observed in mice injected with PBS. It was noted that the responses to cAMP agonists were variable from mouse to mouse in the DSG treated animals. These data suggest that DSG appears to be capable of correcting the defective Cl^- secretion in ΔF508 CF mice *in vivo*.

Although the invention has been described with reference to the disclosed embodiments, those of skill in the art will understand that, using no more than routine experimentation, various modifications can be made without departing from the spirit of the invention. Such equivalents are intended to be encompassed within the scope of the following claims.

What is claimed is:

1. A method for treating defective chloride ion transport in a subject having cystic fibrosis said method comprising:

transporting mutant cystic fibrosis transmembrane regulator (CFTR) protein to the plasma membrane of a cystic fibrosis (CF)-associated cell of said subject wherein said mutant CFTR mediates chloride ion transport in the CF-associated cell of said subject.

2. The method of claim 1 further comprising administering to said subject an agent that modulates chaperone protein binding.

3. The method of claim 2 wherein said chaperone protein is hsp70.

4. The method of claim 2 wherein said agent deoxyspergualin (DSG).

5. The method of claim 1 wherein the CF-associated cell is an epithelial cell.

6. The method of claim 5 wherein the epithelial cell is an airway epithelial cell.

7. A packaged drug comprising:

a container holding an agent for treating defective chloride ion transport in a subject having cystic fibrosis; and

instructions for administering to said subject the agent for treating defective chloride ion transport in said subject.

8. The packaged drug of claim 7 wherein said agent is selected from compounds that interfere with and/or modulate the functioning of molecular chaperone proteins thereby allowing the mutant CFTR protein to escape from the ER, proceed to the plasma membrane and provide functional cAMP-responsive Cl^- channels.

9. The packaged drug of claim 8 wherein said agent is deoxyspergualin and analogs thereof.

10. A method for generating chloride channels in a cystic fibrosis (CF)-associated cell, the method comprising:

contacting said cell with an amount of an agent effective to mobilize mutant cystic fibrosis transmembrane regulator protein in said cell such that said mutant cystic fibrosis transmembrane regulator protein is transported to the plasma membrane of said cell and generates chloride channels therein.

11. The method of claim 10 wherein the CF-associated cell is an epithelial cell.

12. The method of claim 11 wherein the epithelial cell is an airway epithelial cell.

13. The method of claim 10 wherein said agent is deoxyspergualin and analogs thereof.

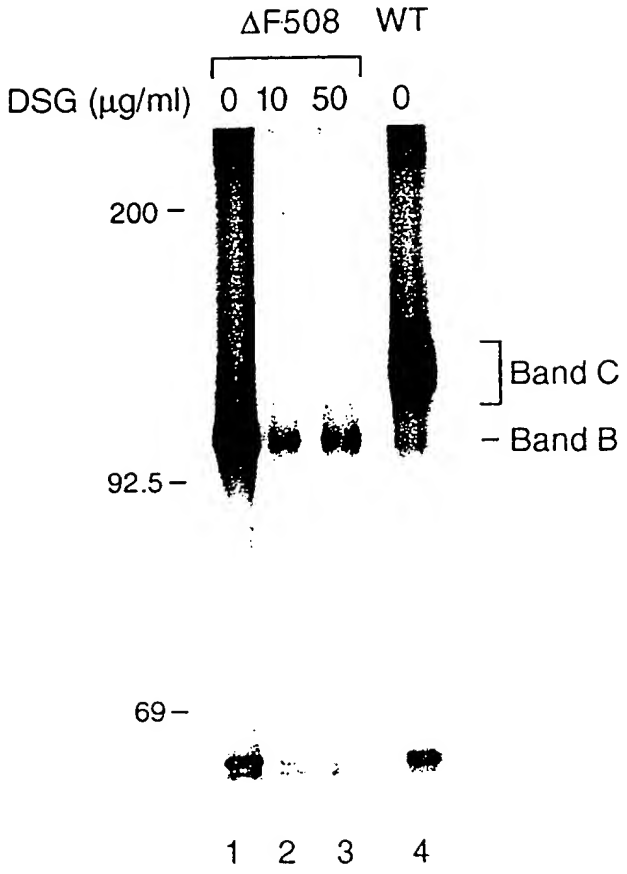


FIG. 1A

SPQ Analysis of C127-ΔF508 Cells Treated with DSG

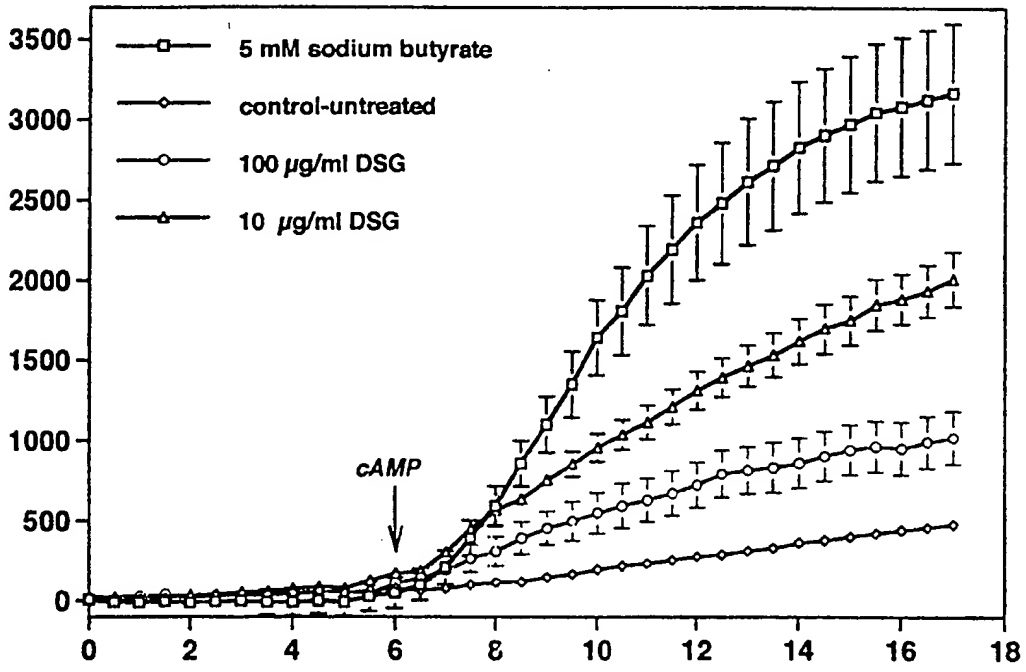


FIG. 1B

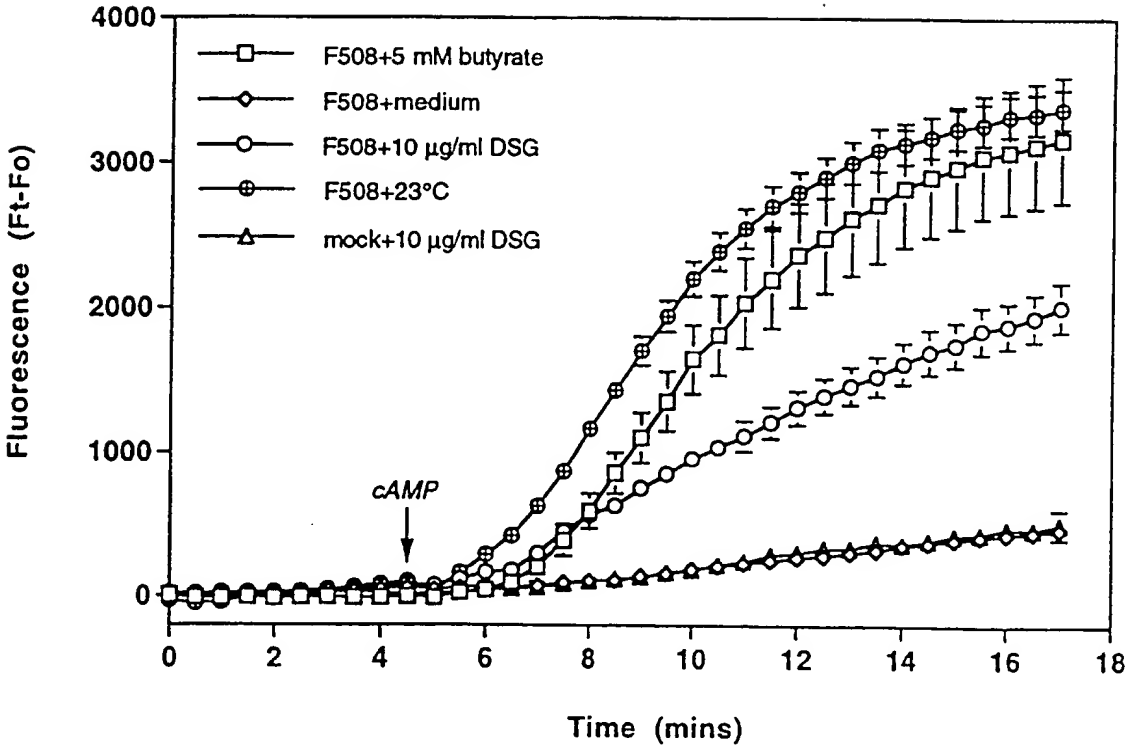

4000
3000
2000
1000
0
Fluorescence (Ft-Fo)
F508+5 mM butyrate
F508+medium
F508+10 µg/ml DSG
F508+23°C
mock+10 µg/ml DSG
cAMP
0
2
4
6
8
10
12
14
16
18
Time (mins)

FIG. 1C

SPQ Analysis of C127-ΔF508 Cells Treated with DSG

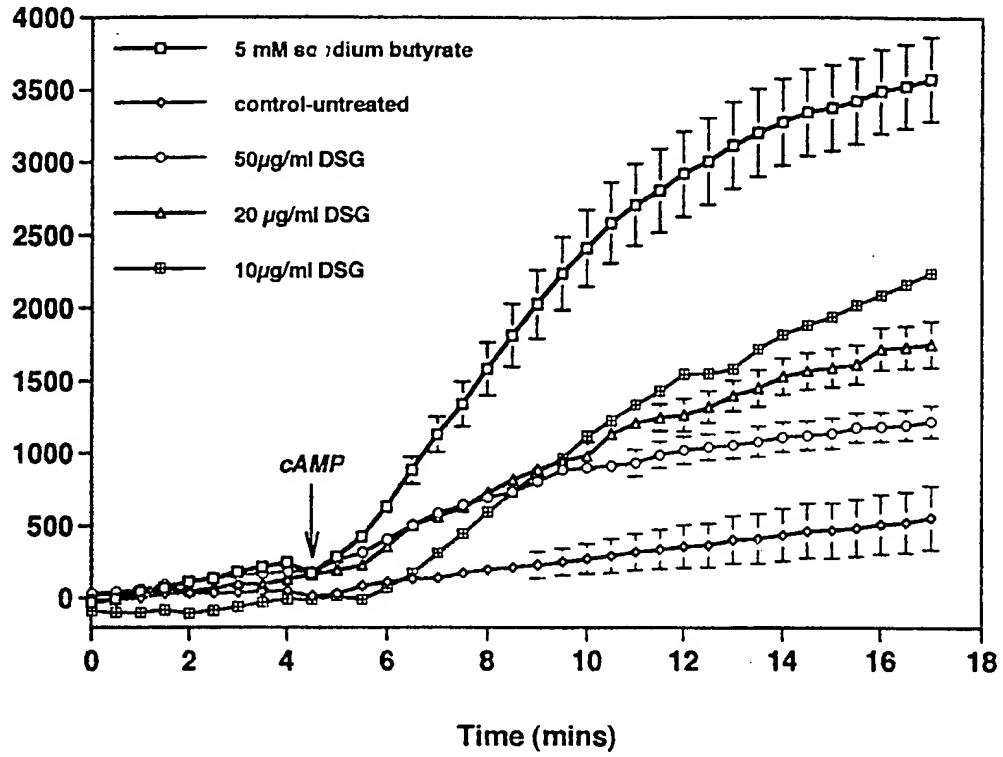


FIG. 1D

Summary of DSG Treated C127-ΔF508 Cells

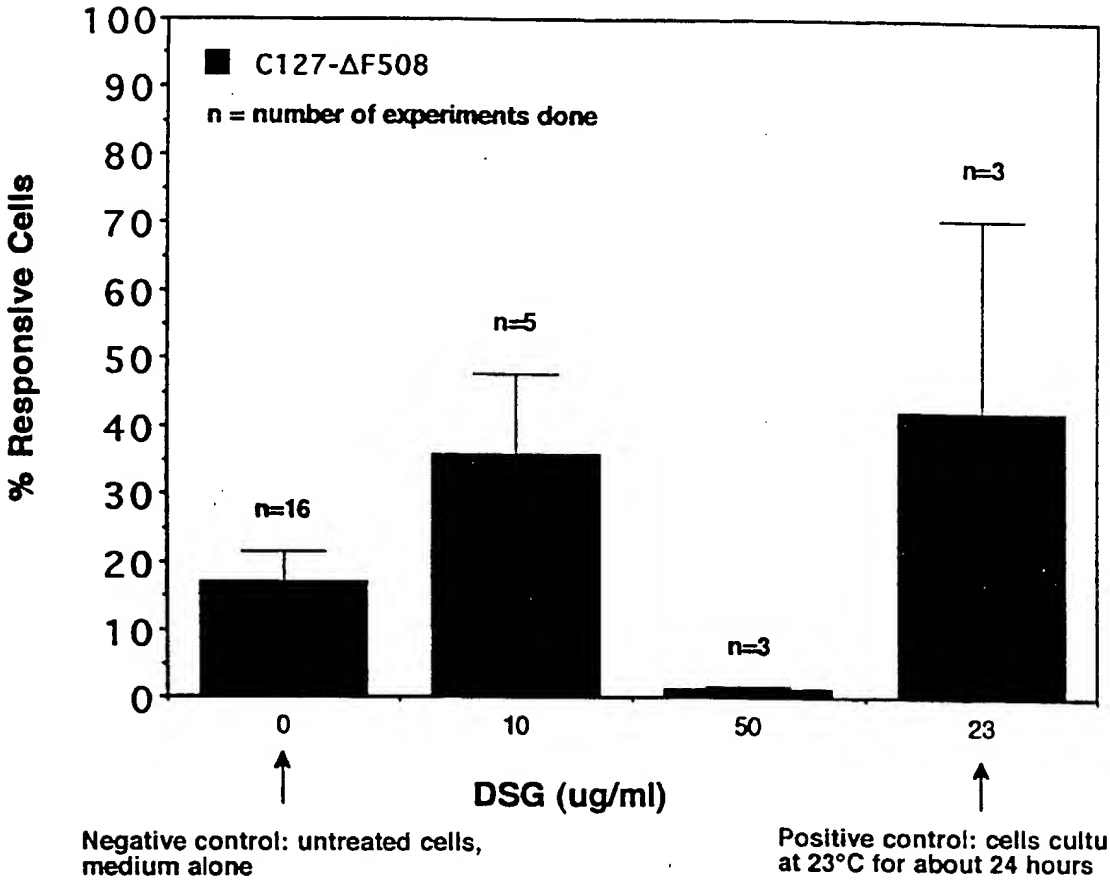
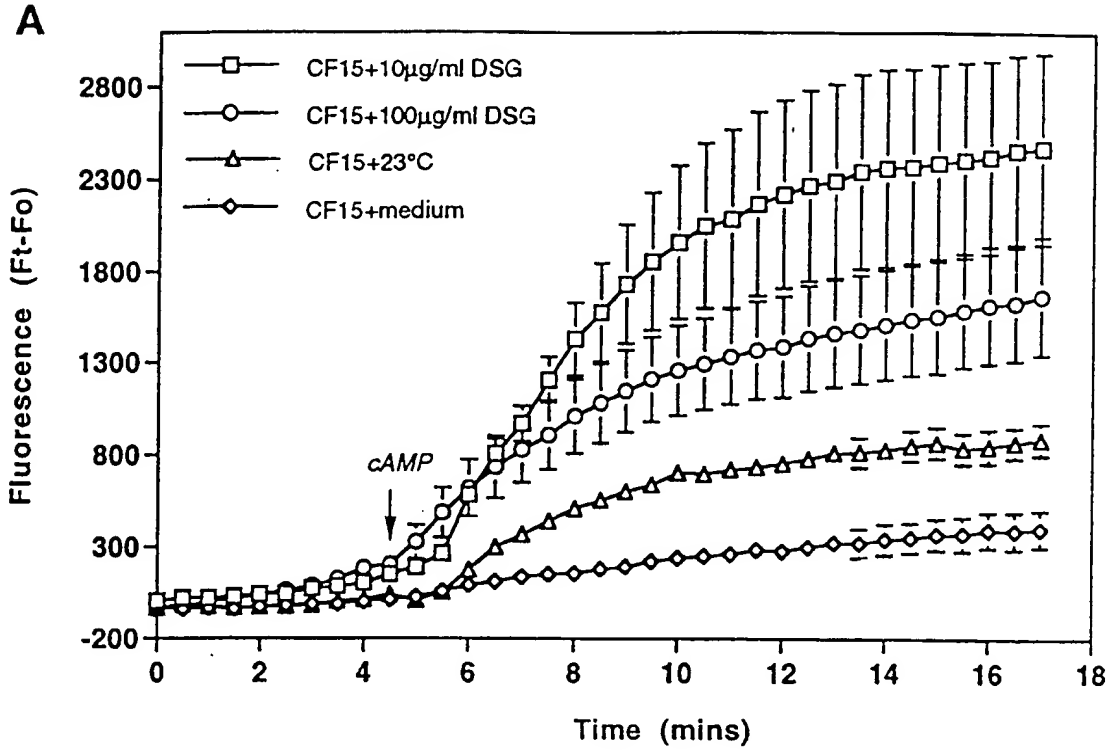
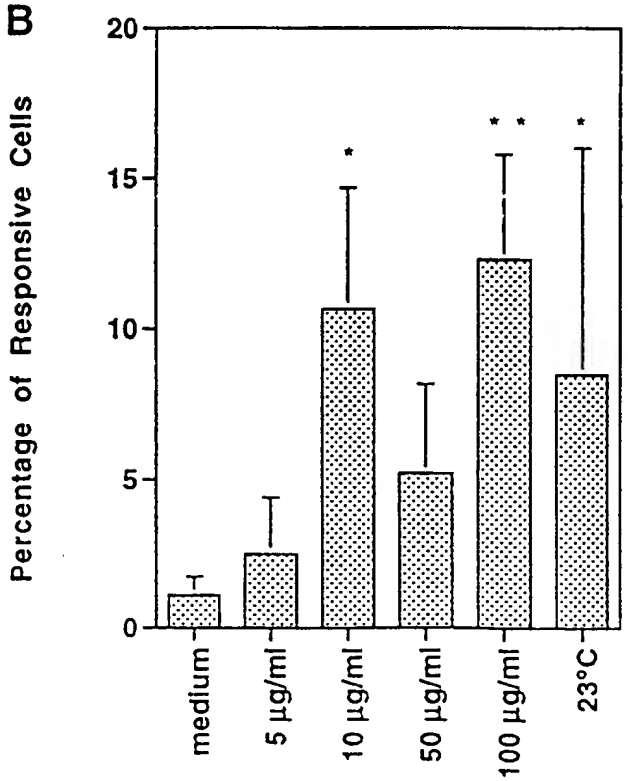


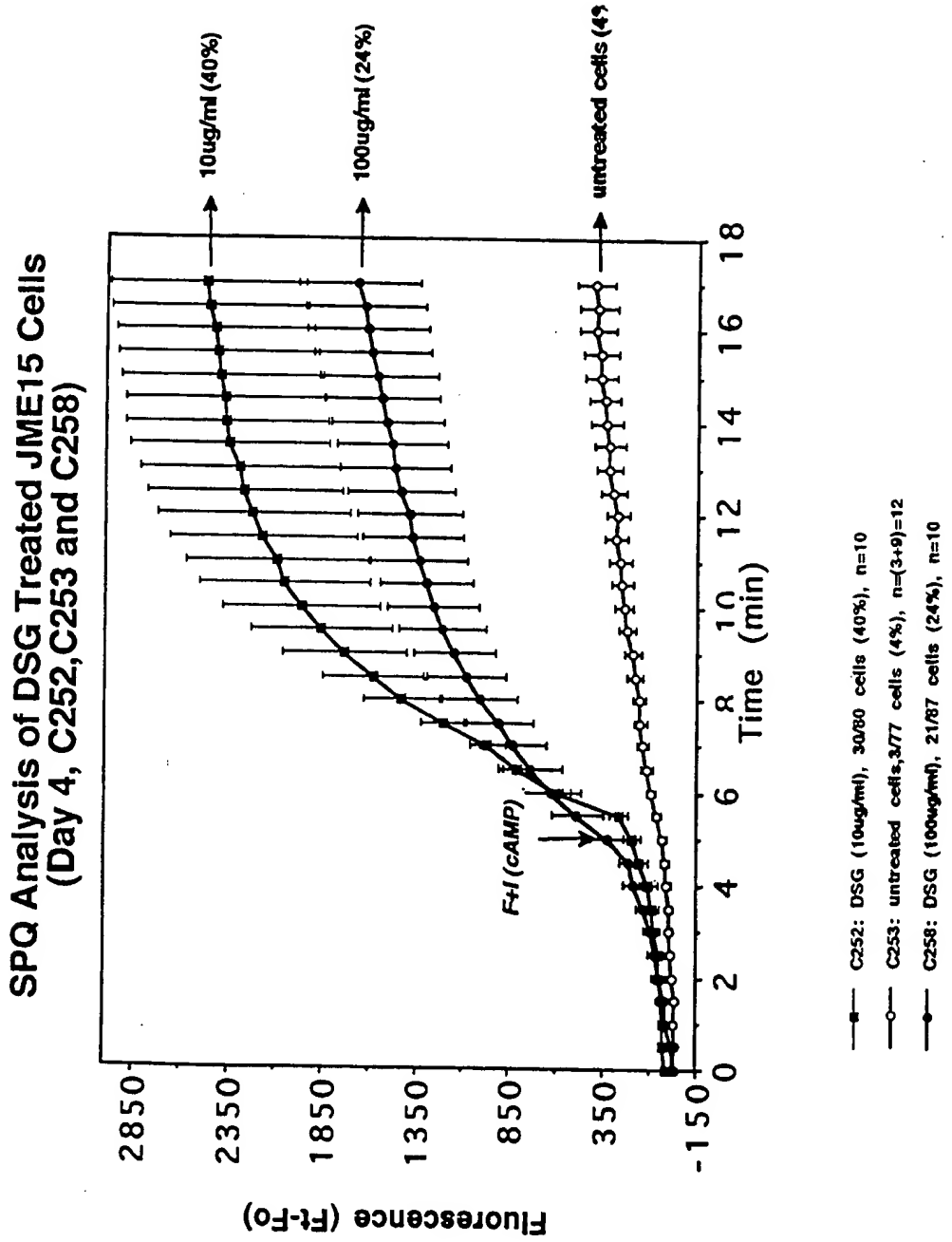
FIG. 1E

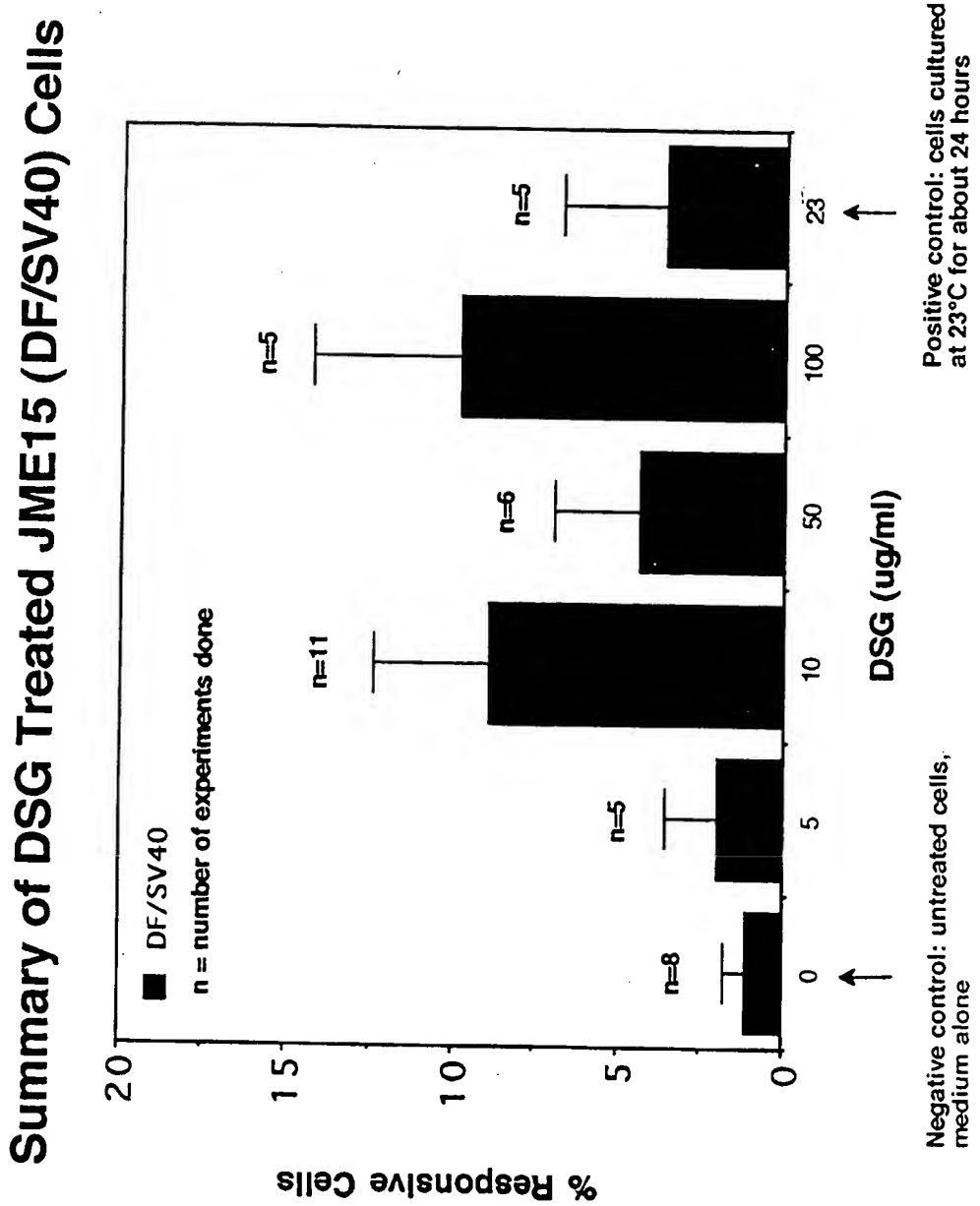
A

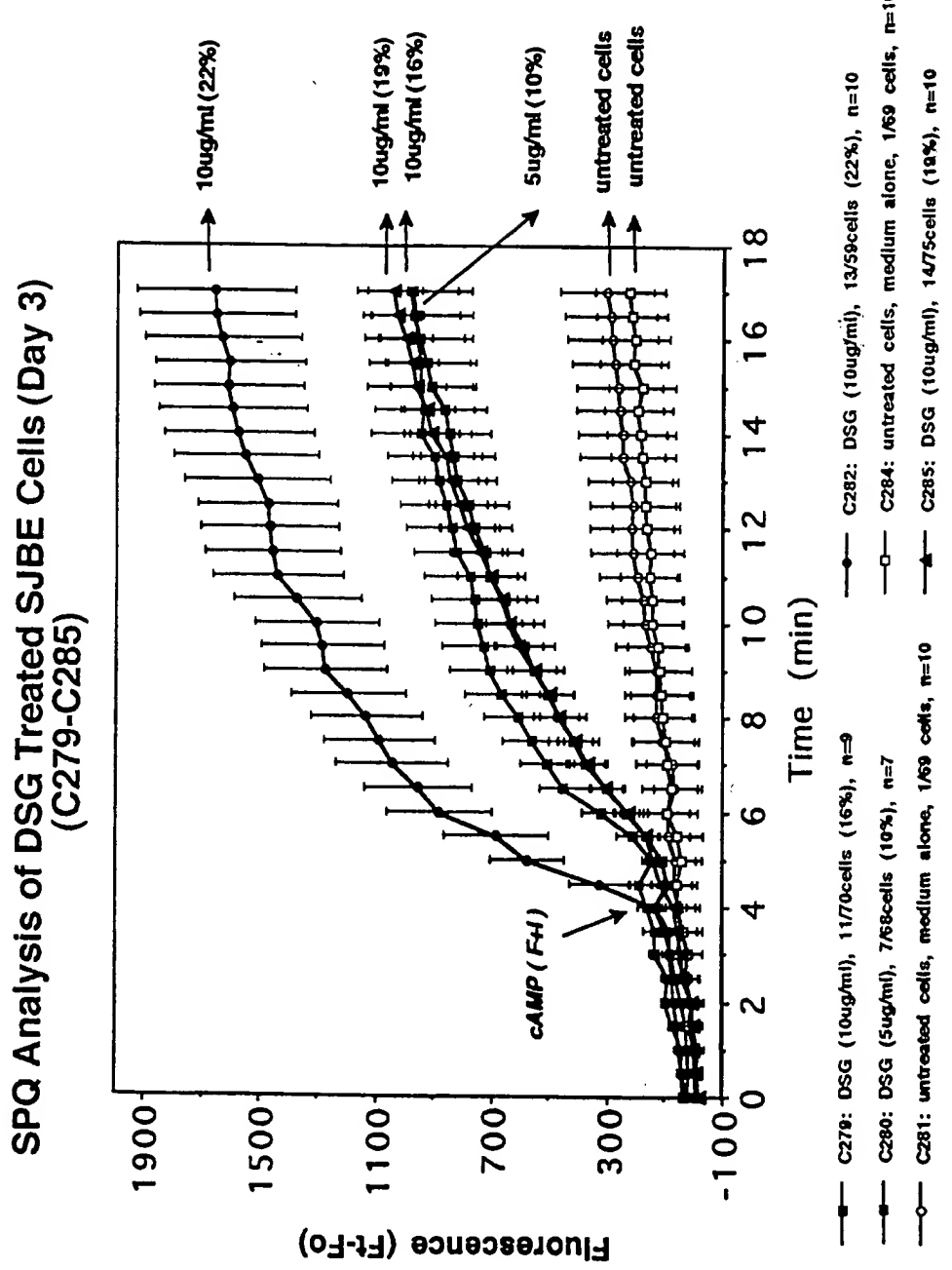

CF15+10µg/ml DSG
CF15+100µg/ml DSG
CF15+23°C
CF15+medium
cAMP
Fluorescence (Ft-Fo)
2800
2300
1800
1300
800
300
-200
0
2
4
6
8
10
12
14
16
18
Time (mins)

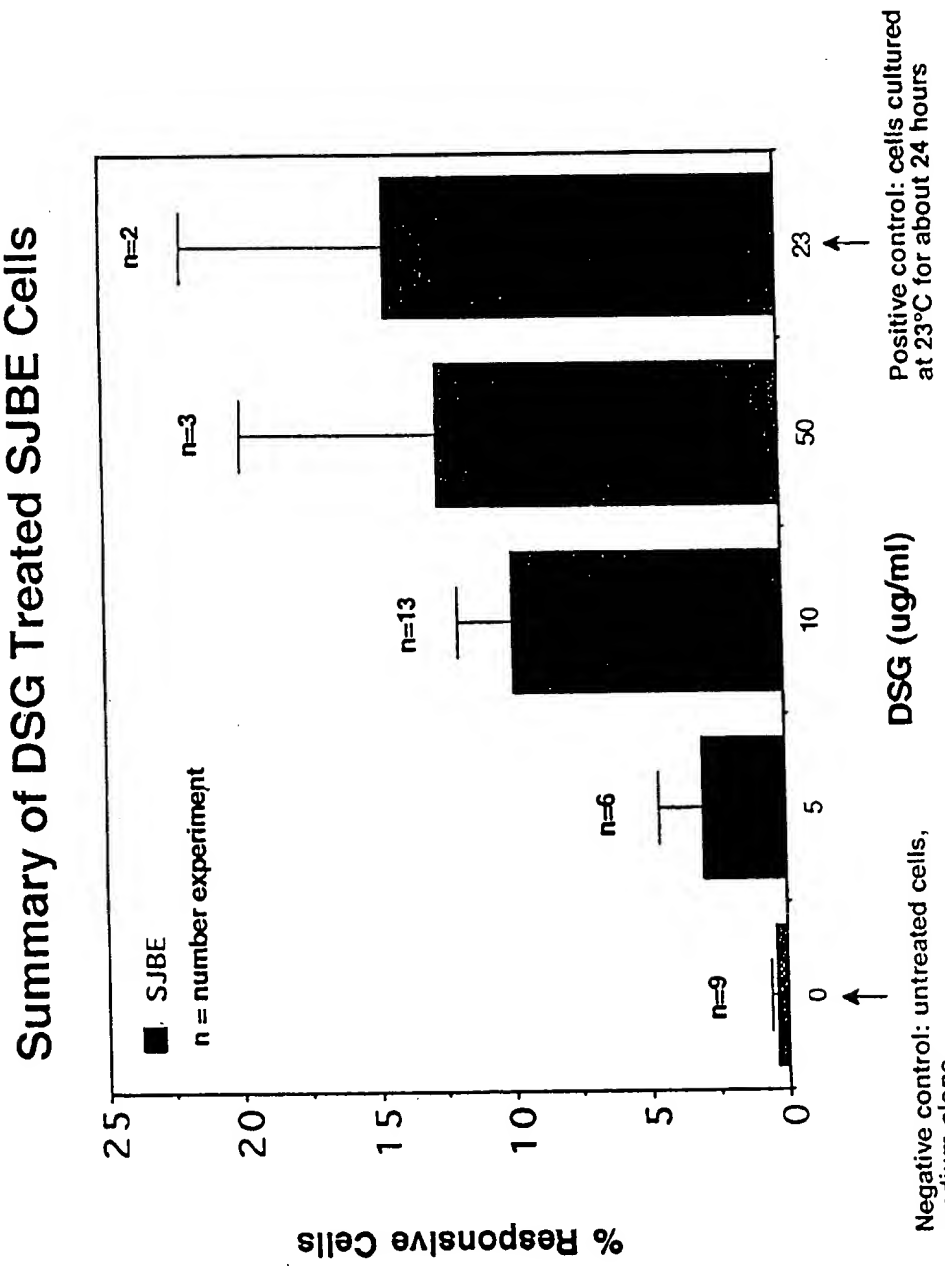
B


Percentage of Responsive Cells
20
15
10
5
0
*
* *
*
medium
5 µg/ml
10 µg/ml
50 µg/ml
100 µg/ml
23°C


SPQ Analysis of DSG Treated JME15 Cells
(Day 4, C252,C253 and C258)
10ug/ml (40%)
100ug/ml (24%)
untreated cells (4%
F+I (cAMP)
Fluorescence (Ft-Fo)
2850
2350
1850
1350
850
350
-150
0
2
4
6
8
10
12
14
16
18
Time (min)
C252: DSG (10ug/ml), 30/80 cells (40%), n=10
C253: untreated cells,3/77 cells (4%), n=(3+9)=12
C258: DSG (100ug/ml), 21/87 cells (24%), n=10


Summary of DSG Treated JME15 (DF/SV40) Cells
DF/SV40
n = number of experiments done
% Responsive Cells
20
15
10
5
0
n=8
n=5
n=11
n=6
n=5
n=5
0
5
10
50
100
23
DSG (ug/ml)
Negative control: untreated cells, medium alone
Positive control: cells cultured at 23°C for about 24 hours


SPQ Analysis of DSG Treated SJBE Cells (Day 3)
(C279-C285)
Fluorescence (Ft-Fo)
1900
1500
1100
700
300
-100
0
2
4
6
8
10
12
14
16
18
Time (min)
10ug/ml (22%)
10ug/ml (19%)
10ug/ml (16%)
5ug/ml (10%)
untreated cells
untreated cells
cAMP (Fi)
C279: DSG (10ug/ml), 11/70cells (16%), n=9
C280: DSG (5ug/ml), 7/68cells (10%), n=7
C281: untreated cells, medium alone, 1/69 cells, n=10
C282: DSG (10ug/ml), 13/59cells (22%), n=10
C284: untreated cells, medium alone, 1/69 cells, n=1
C285: DSG (10ug/ml), 14/75cells (19%), n=10


Summary of DSG Treated SJBE Cells
SJBE
n = number experiment
% Responsive Cells
25
20
15
10
5
0
n=9
n=6
n=13
n=3
n=2
0
5
10
50
23
DSG (ug/ml)
Negative control: untreated cells, medium alone
Positive control: cells cultured at 23°C for about 24 hours

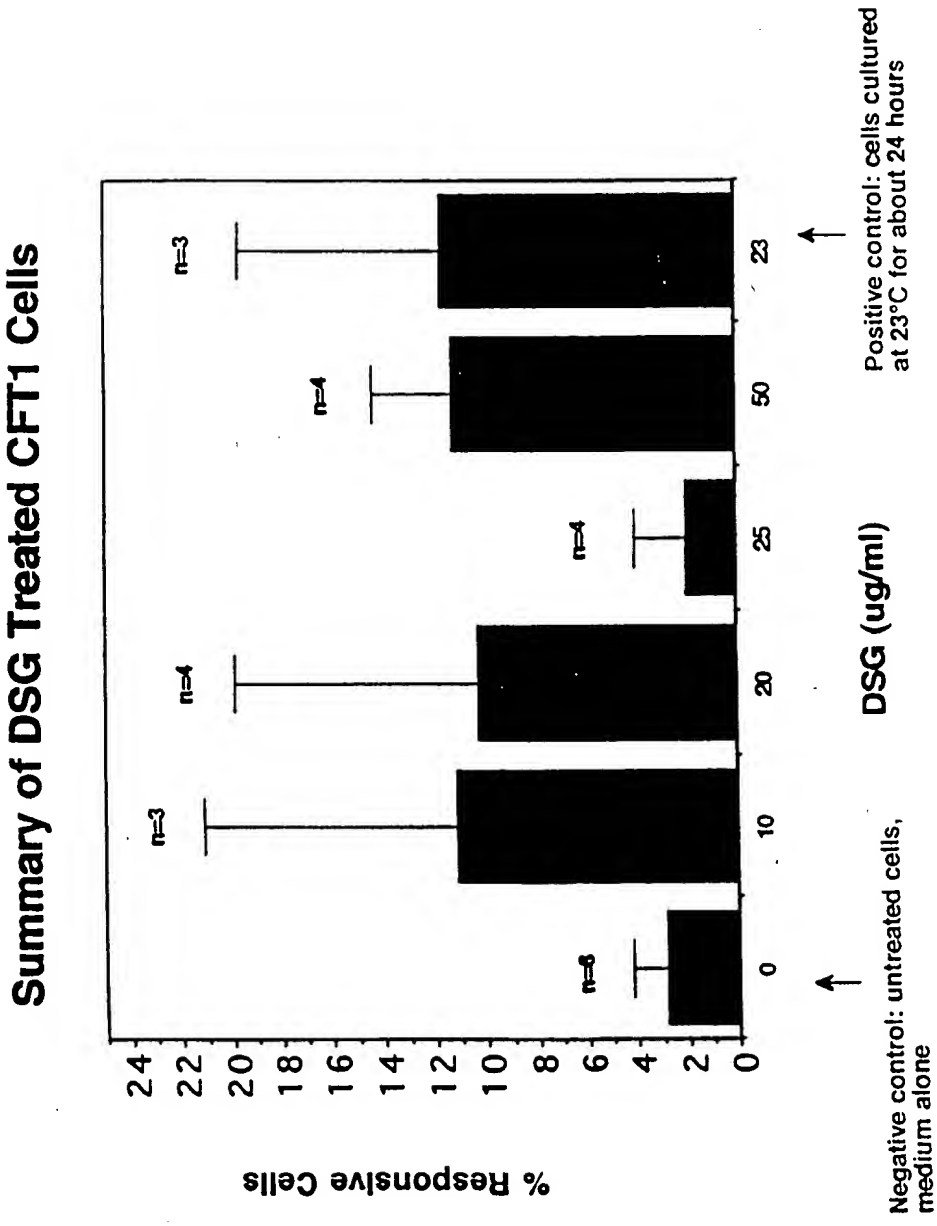

Summary of DSG Treated CFT1 Cells
% Responsive Cells
24
22
20
18
16
14
12
10
8
6
4
2
0
n=3
n=4
n=4
n=4
n=3
0
10
20
25
50
23
DSG (ug/ml)
Negative control: untreated cells, medium alone
Positive control: cells cultured at 23°C for about 24 hours
CFT1 cells
n = number experiment

Fig. 7

Summary of DSG Treated LLCPK1-ΔF508 Cells

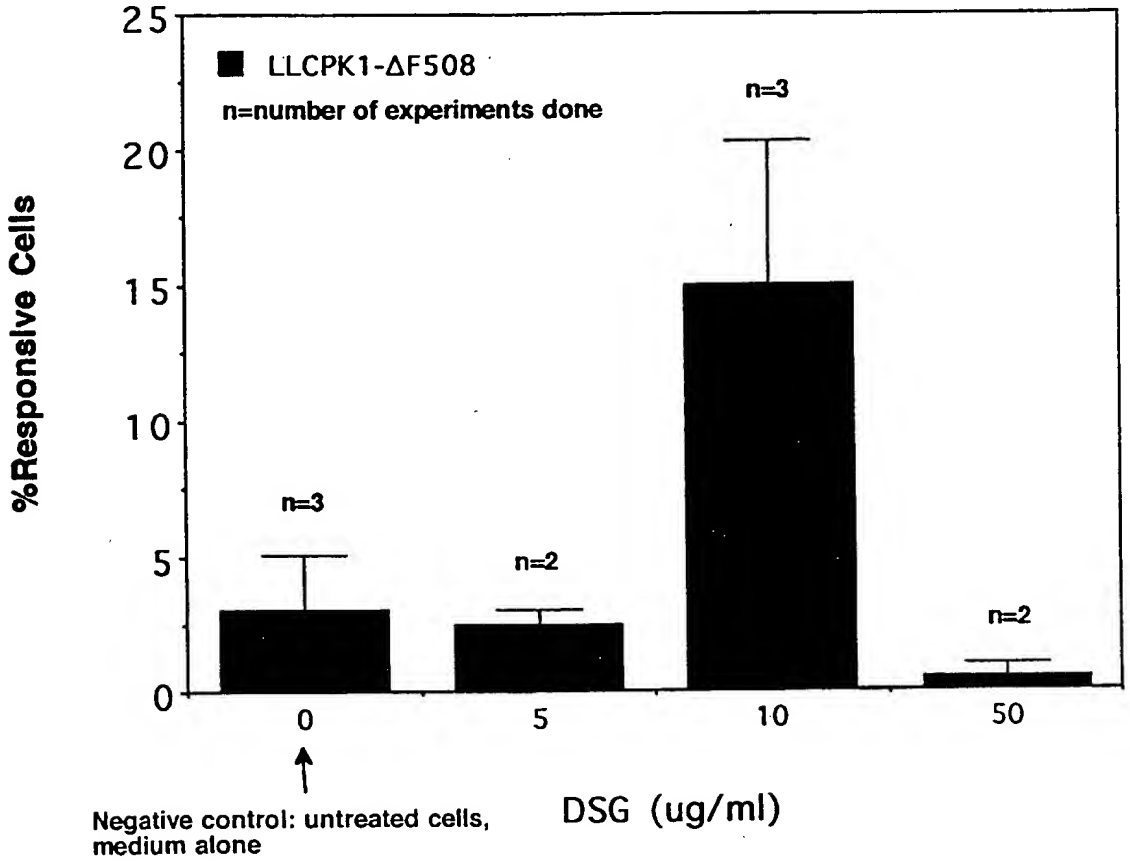
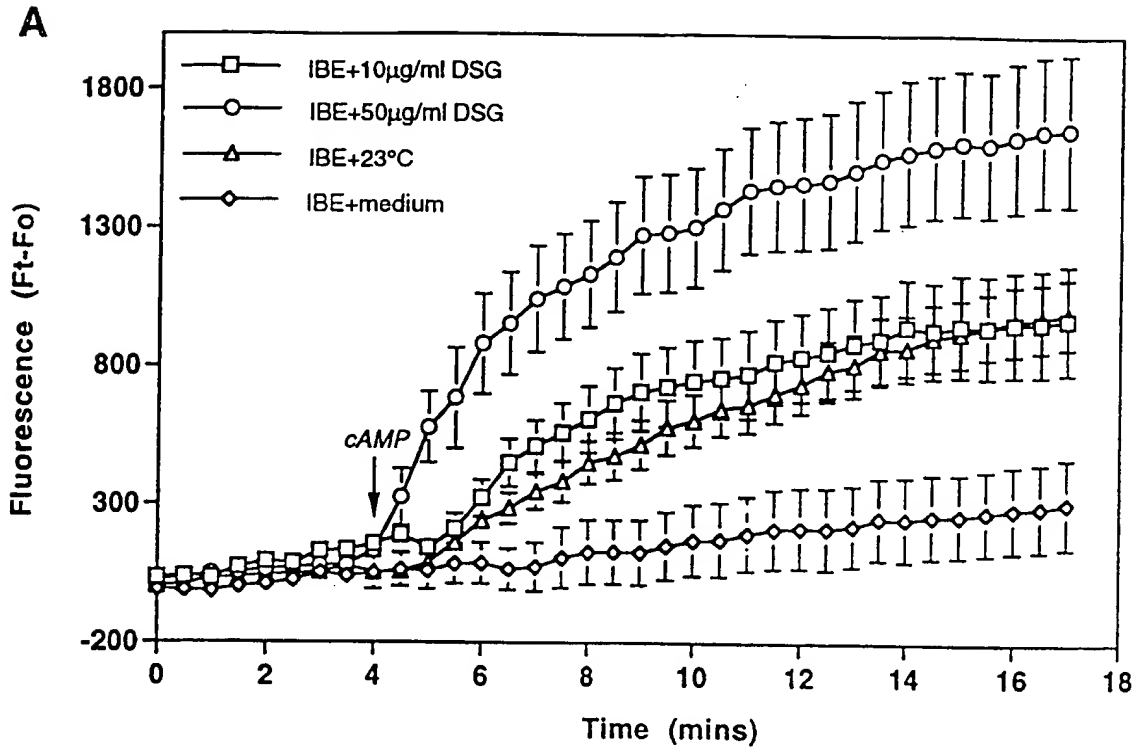
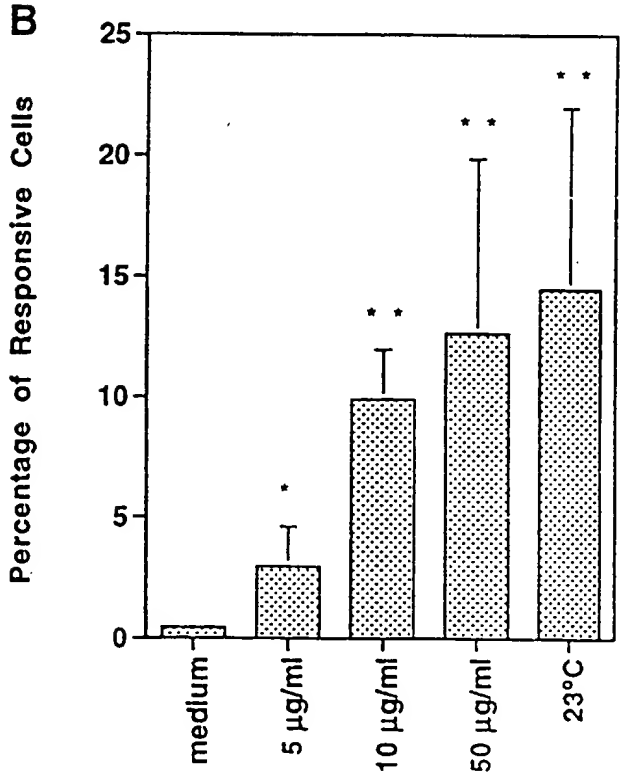


Fig. 8


A
IBE+10µg/ml DSG
IBE+50µg/ml DSG
IBE+23°C
IBE+medium
cAMP
Fluorescence (Ft-Fo)
1800
1300
800
300
-200
0
2
4
6
8
10
12
14
16
18
Time (mins)


B
Percentage of Responsive Cells
25
20
15
10
5
0
medium
5 µg/ml
10 µg/ml
50 µg/ml
23°C

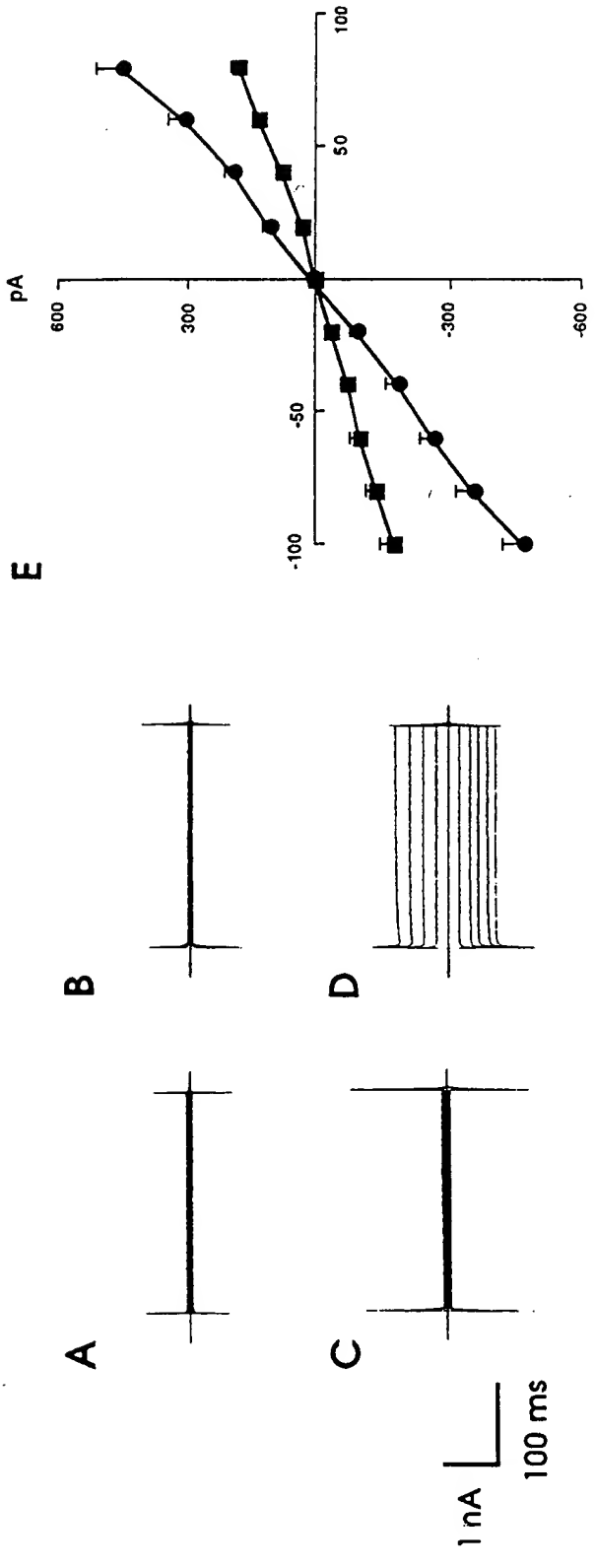


FIG. 10

SPQ Analysis of Tracheobronchial Epithelial Cells Isolated from ΔF508 Mice Treated with DSG

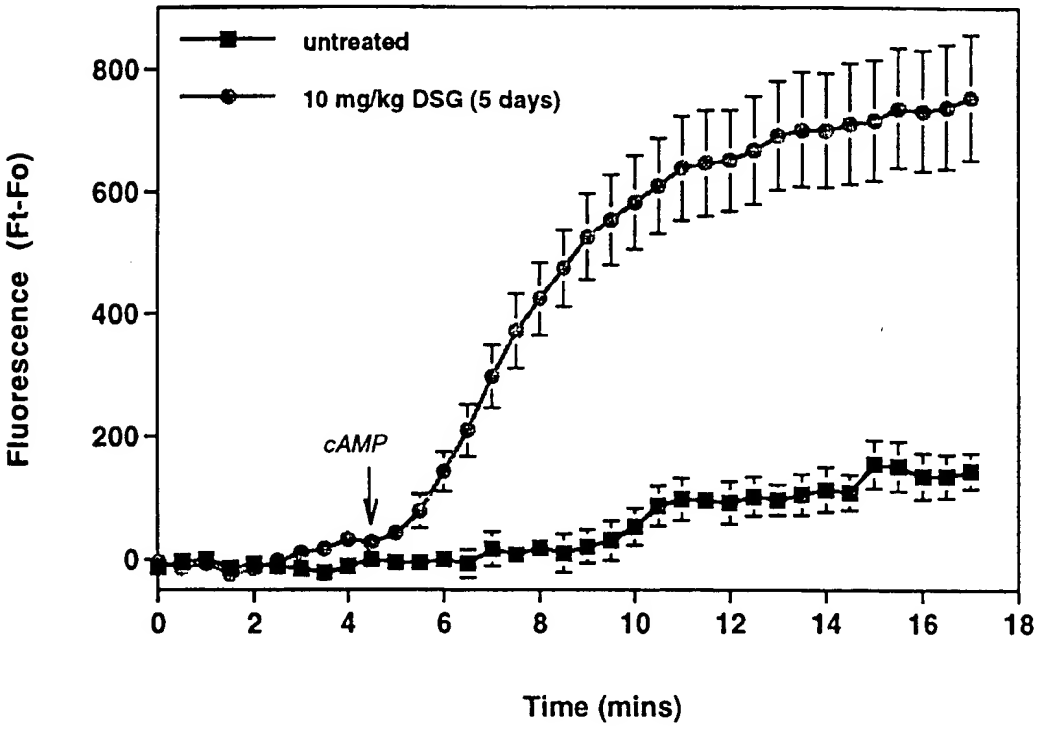


FIGURE 11

INTERNATIONAL SEARCH REPORT

Inte. ɔnal Application No
PCT/US 98/03672

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 04671 A (GENZYME CORP ;UNIV IOWA RES FOUND (US)) 3 March 1994 see page 7, line 36 - page 8, line 16; claims 1-38 ---	1-3,5-8, 10,11
X	CHENG S.H. ET AL : "Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 268, no. 4 pt 1, 1995, pages L615-L624, XP002064541 see page L622, left-hand column, paragraph 2 - page L623 --- -/--	1-3,5-8, 10,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
11 May 1998	27/05/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter .nal Application No
PCT/US 98/03672

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SMITH A.E.: "Treatment of cystic fibrosis based on understanding CFTR" JOURNAL OF INHERITED METABOLIC DISEASE, vol. 18, no. 4, 1995, pages 508-516, XP002064542 see page 510 ---	1-3,5-8, 10,11
X	EP 0 256 385 A (BEHRINGWERKE AG) 24 February 1988 see claims 1,2 ---	7-9
X	WO 96 12406 A (GENETIC THERAPY INC ;TRAPNELL BRUCE C (US); YEI SOONPIN (US); MCCL) 2 May 1996 see page 21, last paragraph; claim 10 ---	7-9
A	NADEAU K. ET AL: "Quantitation of the interaction of the immunosuppressantdeoxyspergualin and analogs with Hsc70 and Hsp90" BIOCHEMISTRY, vol. 33, 1994, pages 2561-2567, XP002064543 cited in the application see abstract -----	1-11

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 98/03672

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9404671 A	03-03-94	US 5674898 A	07-10-97
		AU 684049 B	04-12-97
		AU 5092793 A	15-03-94
		CA 2143306 A	03-03-94
		EP 0659211 A	28-06-95
		JP 8500596 T	23-01-96
EP 0256385 A	24-02-88	DE 3626306 A	11-02-88
		AT 130192 T	15-12-95
		AU 605494 B	17-01-91
		AU 7633987 A	04-02-88
		CA 1305424 A	21-07-92
		DE 3751597 D	21-12-95
		DK 400887 A	03-02-88
		ES 2081279 T	01-03-96
		JP 2610621 B	14-05-97
		JP 63041419 A	22-02-88
		KR 9513453 B	08-11-95
		PT 85454 B	29-06-90
		US 4847299 A	11-07-89
WO 9612406 A	02-05-96	EP 0804076 A	05-11-97